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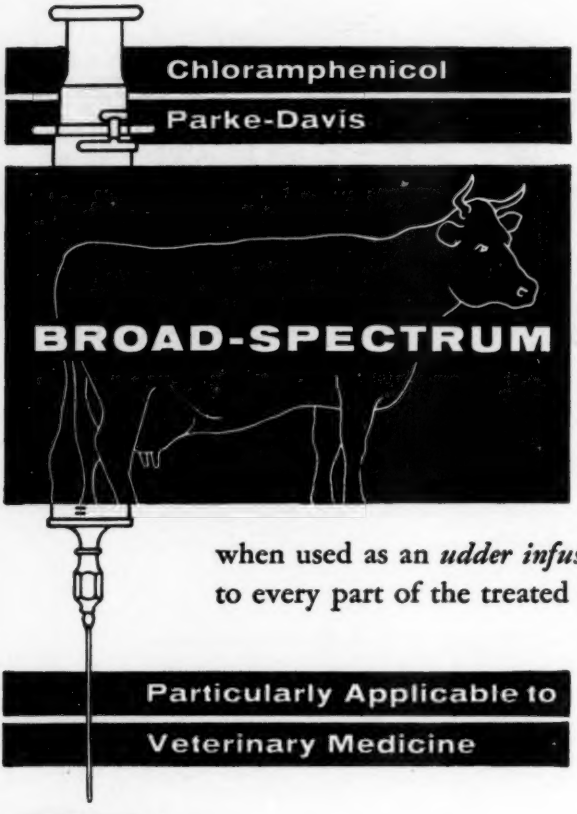

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Volume XX May, 1959 Number 76

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VOL. XX MAY, 1959 No. 76

Studies on Urolithiasis. III. The Control by Force Feeding Sodium Chloride

R. H. UDALL, D.V.M., Ph.D.

Fort Collins, Colorado

A NUMBER of rations have been described which have resulted in the production of urinary calculi and its clinical manifestation, called "waterbelly." In general, phosphorus has been most frequently considered as the cause,[3,6] although cottonseed meal has been implicated without reference to its phosphorus content.[4] It is apparent that hypotheses about the causative agents, based on the mineral components of the ration and the resulting changes in the excretion of these materials in the urine, have not led to useful results in the control of the disorder.[7]

It has been reported that the feeding of milo increases the amounts of calculi that can be recovered from the bladders of steers as compared with steers fed corn.[1,5]

The use of force-fed sodium chloride has been reported to successfully control the incidence of calculi in fattening lambs.[2] A level of 10 per cent of the total dry matter was used and the whole ration was pelleted, which insured the consumption of the required amount of salt.

Reports on waterbelly indicate that it occurs most frequently during the winter months, and ranchers report that it appears to be associated with storms and shifts in the temperature. This observation has only rarely been mentioned in the literature.[7]

EXPERIMENTAL PROCEDURE

It was proposed in this experiment to determine the effect of sex, type of concentrate fed, and the force feeding of sodium chloride on the incidence of urinary calculi.

The design was a randomized pen feeding trial with a factorial arrangement of the treatments: males versus females; corn versus milo; and 10 per cent sodium chloride versus no sodium chloride, force fed in a pelleted ration. Ninety-six lambs, half wethers and half ewes, were randomly placed in four lots of 12 males and 12 females each. The feed (table 1) was continuously available in self feeders. The feeding period started on Jan. 14, 1957, and the lambs were slaughtered on April 30, after 106 days on feed. At slaughter, the urinary bladders and kidneys were recovered and subsequently examined for calculi. The bladders were opened over a black photographic tray and washed to determine whether calculi were present. The kidneys were sliced in half longitudinally, the major and minor calyces opened to the surface with scissors and washed into a black photographic tray to recover any calculi present. Usually, the amount recovered from a pair of kidneys was too small to weigh, so all renal calculi were dissolved

TABLE 1—Feed Composition of the Different Rations

Ingredient	No NaCl		10% NaCl	
	Corn (%)	Milo (%)	Corn (%)	Milo (%)
Corn	56.0	.....	56.0	.....
Milo	.....	56.0	.....	56.0
Cottonseed meal	6.0	6.0	6.0	6.0
Molasses	8.0	8.0	8.0	8.0
Ground alfalfa	20.0	20.0	20.0	20.0
Sugar	10.0	10.0	.....	.....
Salt	.....	.....	10.0	10.0
ANALYTICAL COMPOSITION				
Moisture (%)	7.5	8.4	9.4	8.6
Protein (%)	12.4	12.9	11.5	12.6
Carotene (mg./lb.)	2.3	2.8	2.8	2.8
Ash (%)	3.7	3.7	13.5	13.7
Calcium (%)	0.34	0.34	0.42	0.37
Phosphorus (%)	0.32	0.33	0.22	0.32

From the College of Veterinary Medicine and the Agricultural Experiment Station, Colorado State University, Fort Collins.

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in hydrochloric acid, made up to known volumes, and the total amount of phosphorus present determined as a quantitative measure of the relative amount of calculi recovered from each pair of kidneys.

Lambs that showed signs of urethral obstructions during the feeding period were euthanatized so that a positive diagnosis could be made.

RESULTS AND DISCUSSION

The results of the trial, using the occurrence of calculi in the urinary tract to measure the effect of the treatments, are summarized (table 2). In order to increase the precision of measurement, the incidence of bladder calculi was combined with the deaths resulting from waterbelly in comparing the effects of the dietary factor, corn versus milo, since the presence of calculi in the bladder imposed a high probability of obstruction of the urethra if they were carried out during urination.

TABLE 2—The Effects of Sex and Dietary Factors on the Incidence of Calculosis in Lambs

	No NaCl				10% NaCl			
	Corn		Milo		Corn		Milo	
Item	M	F	M	F	M	F	M	F
Deaths	2	0	5	0	0	0	0	0
Bladder calculi	0	0	2	0	0	0	0	0
No. kidney pairs containing calculi	4	9	8	6	0	0	0	0
Total phosphorus (mg). in renal calculi	6.1	5.8	3.4	1.7	0.0	0.0	0.0	0.0
Av. total gain (lb.)	52.1	42.4	51.3	39.6	50.3	45.8	49.4	42.9
Dressing percentage	51.7	50.7	52.4	50.9	49.9	49.6	50.9	50.7
Lb. feed/lb. gain	7.5		9.8		7.3		7.7	
No choice carcasses	21		17		22		22	

There were no deaths and no bladder nor renal calculi among the 48 lambs fed 10 per cent sodium chloride in the ration, as compared to 5 deaths, two bladders containing appreciable quantities of calculi, and a 52 per cent incidence of renal calculi among the 48 lambs given no salt. It seems reasonable to hypothesize that the use of 10 per cent sodium chloride prevented the formation of calculi by forcing the animals to drink large quantities of water, which in turn resulted in the excretion of large volumes of urine. The large volume of urine would result in a dilution of those components which, it has been hypothesized,[8] promote the deposition of minerals as calculus material under conditions of a higher concentration, provided the total daily excretion was not affected by the ration. There was no evidence in this trial that the 10 per cent level of sodium chloride had any effect upon the weight gain, dressing percentage, feed efficiency, or carcass grade as shown by statistical analysis of these data.

TABLE 3—Comparison of Amounts of Bladder Calculi Recovered from Lambs when Fed Milo or Corn

Animal No.	Date euthanatized	Ration (no salt)	Bladder calculi (Gm.)
526	3/12/57	milo	0.61
257	3/19/57	corn	1.50
277	4/10/57	milo	0.74
255	4/11/57	milo	lost*
527	4/14/57	milo	1.64
285	4/15/57	corn	0.05
233	4/19/57	milo	1.12

* Bladder ruptured before euthanasia.

Using *chi* square to compare the effects of milo with corn as sources of concentrates, there was a significantly higher incidence ($P<0.02$) of deaths and bladder calculi associated with the milo feeding, though there was no difference in the amounts of renal calculi between the two grains. This raises the question of the relation between renal calculi and bladder calculi, and whether bladder calculi are formed locally or whether they are stones shed by the kidneys. Since the incidence of deaths and bladder calculi was significantly different in the sheep fed the two grains, whereas there was no difference in the incidence of renal calculi, it is probable that under most conditions large amounts of calculi form in the bladder and are carried into the urethra, where they lodge and form obstructions. This does not preclude the possibility of renal calculi ultimately causing an obstruction of the urethra. Substantial amounts of small sand-like calculi were found in the bladder (table 3) of most of the lambs dying from waterbelly, which indicates that the stones formed rapidly in the urine in the bladder,

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following the imposition of some additional stress which brought about conditions favorable for mineral deposition. Five of the 7 lambs were euthanatized between April 10 and 19 (table 3), immediately following a severe spring snow storm on April 9, accompanied by cold weather which continued until April 16. This emphasizes the relationship between the occurrence of calculi, with obstruction of the urethra, and the incidence of weather stress. It leads to the postulation that cold weather probably induces a diuresis accompanied by a reduced water intake, followed by dehydration and excretion of a more concentrated urine. A more concentrated urine would appear to favor mineral deposition, for the mechanism which has been postulated to promote mineral deposition [8] would then occur in greater concentrations.

The incidence of calculi in the kidneys was the same for males and females, indicating no difference between sexes, and that there is no direct association of endocrine secretions and calculous formation.

SUMMARY

The force feeding of 10 per cent sodium chloride as an integral part of a pelleted ration prevented the formation of both bladder and renal calculi. It was postulated that the increased water consumption necessitated by the salt resulted in the excretion of a more dilute urine with respect to a mechanism promoting mineral deposition, thus preventing calculous formation.

There was a higher incidence of clinical urolithiasis with the feeding of milo than with corn, but no difference in the amounts of renal calculi.

There was no difference between males and females in the amounts of renal calculi.

The death of 5 of the 7 lambs from clinical urolithiasis immediately following a severe spring snow storm indicated that climatic stress may be an important causative factor in clinical urolithiasis.

References

[1] Black, W. H., Ellis, N. R., Jones, J. M., and Keating, F. E.: The Relation Between Urinary Calculi and the Feeding of Grain Sorghums in Steer Fattening Rations in the Southern Great Plains. U.S.D.A. Tech. Bull. 945, (1947): 14.

[2] Elam, C. J., Schneider, B. H., and Ham, W. E.: Dietary Influence on the Formation of Urinary Calculi in Sheep. Proc. Western Sect., Am. Soc. Anim. Prod., *6*, (1955): 223–225.

[3] Elam, C. J., Schneider, B. H., and Ham, W. E.: Experimentally Produced Urinary Calculi in Lambs. J. Anim. Sci., *15*, (1956): 800–810.

[4] Harris, L., and Alexander, M. A.: Beet Top Silage, a Good Feed for Lambs. Nebraska Exper. Sta. Quart., *2*, (1953): 14–16.

[5] Jones, J. H., Keating, F. E., Ellis, N. R., and Crookshank, H. R.: Urinary Calculi in Fattening Steers. Texas Agric. Exper. Sta., Progress Report 1570, Cattle Series 109.

[6] Lindley, C. E., Tayson, E. R., Ham, W. E., and Schneider, B. H.: Urinary Calculi in Sheep. J. Anim. Sci., *12*, (1953): 704–714.

[7] Newsom, I. E.: Urinary Calculi with Special Reference to Sheep. J.A.V.M.A., *92*, (April, 1938): 495–502.

[8] Udall, R. H., Deem, A. W., and Maag, D. D.: Studies on Urolithiasis. I. Experimental Production in Steers Associated with Feeding. Am. J. Vet. Res., *19*, (Oct., 1958): 824.

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Studies on Urolithiasis. IV. The Effects of the Ration on the Predisposition as Measured by the Urinary Mucoproteins

R. H. UDALL, D.V.M., Ph.D.

Fort Collins, Colorado

THE NUMEROUS HYPOTHESES which have been advanced to explain the mechanism of urinary calculus formation have been summarized by Randall.[10] Most of them have been developed around the concept that dietary minerals or mineral imbalances lead to a change in the mineral structure of the urine and, thence, to deposition of the minerals as crystals in the kidneys or bladder. Such a concept of crystallization lacks a counterpart in other tissues.

Recently, Howard[6] proposed the hypothesis that a mucoprotein matrix is required as a structural foundation for mineral deposition in the urinary tract. A similar mechanism is responsible for the mineralization of the skeleton.[11] A body of evidence that there is an increase in the urinary mucoproteins in human beings with calculous disease has been reported by Boyce *et al.*,[2,3] and they have established the existence of a mucoprotein matrix as part of the structure of a urinary calculus.[4]

Udall *et al.*[12] demonstrated that the calculi from steers fed fattening rations contained 3 to 4 per cent protein and contained hexosamine, indicative of a mucoprotein matrix. This work also indicated that mineral imbalances do not play a significant role in the incidence of calculous disease, but that the feeding of a high concentrate ration increased the predisposition to calculosis as measured by the amounts of mucoproteins in the urine. Several reports[1,15] have indicated that certain grains, such as corn and milo, used commonly in fattening rations, have different effects on the occurrence of calculous disease.

MATERIALS AND METHODS

This trial was designed as a study of the effects of certain rations on the pattern of excretion of the urinary mucoproteins. Rations 1, 2, and 3 were identical with those used for a large-scale feeding trial,[15] during which there were significant differences in the incidence of clinical urolithiasis or "waterbelly" attributable to these rations. The trial was carried out in five successive periods. During the first three periods of 24 days each, rations 1 through 3 (table 1) were fed, in that order. Rations 1 and 2 had been shown to induce appreciable waterbelly, while neither clinical urolithiasis nor renal calculi occurred when lambs were fed ration 3.[15] During the fourth period of 24 days, the lambs were fed chopped, sun-cured alfalfa hay to determine the effect of a legume roughage on the urinary mucoproteins. In the fifth period of ten days, the lambs were shifted back to a concentrated feed by increasing the proportion of concentrate by 2 oz. daily, and decreasing the roughage proportionally to obtain the correlation between increasing amounts of concentrates and the mucoproteins in the urine.

TABLE 1—Feed Composition of the Different Rations

	Period 1 (corn)	Period 2 (milo)	Period 3 (milo/salt)
Corn	56	....	....
Milo	....	56	56
Cottonseed meal	6	6	6
Molasses	8	8	8
Ground alfalfa	20	20	20
Sugar	10	10	....
Salt	....	....	10
ANALYTICAL COMPOSITION			
Moisture (%)	7.5	8.4	8.6
Protein (%)	12.4	12.9	12.6
Carotene (mg./lb.)	2.3	2.8	2.8
Ash (%)	3.7	3.7	13.7
Calcium (%)	0.34	0.34	0.37
Phosphorus (%)	0.32	0.33	0.32

Six wether lambs were maintained in metabolism cages which permitted the collection of consecutive 24-hour urine samples. In order to reduce contamination of the urine, fecal collection sacks were used, and the urine was passed through gauze in a Buchner funnel interposed between the collection pan and the collection bottle which contained a small amount of toluene to preserve the urine. The total volume was measured daily, and a composite of proportional aliquots of three daily samples was frozen for subsequent analysis.

Chemical analyses of the composite samples were made on the phosphotungstic acid-precipitable material for hexosamine by the Elsdon-Morgan reaction[6] and for nitrogen by a micro-Kjeldahl method. The hexosamine was measured as an estimate of the mucopolysaccharide moiety and nitrogen as an estimate of the protein moiety of the mucoprotein complex. As stated in an earlier paper,[12] the hypothesis has been used that the urinary mucoproteins measure the degree of predisposition to calculous disease.

From the College of Veterinary Medicine and the Agricultural Experiment Station, Colorado State University, Fort Collins.

TABLE 2—Effects of the Dietary Factors on the Composition of the Urine in Lambs

Feeding period	No. days	Ration	Total urine volume (ml./24 hr.)	Protein-bound hexosamine: Concentration (μg./ml.)	Protein-bound hexosamine: Total excretion (mg./24 hr.)	Protein nitrogen: Concentration (μg./ml.)	Protein nitrogen: Total excretion (mg./24 hr.)
1	24	Corn	624.0	35.2	21.1	373.0	221.0
2	24	Milo	650.0	40.3	25.7	341.0	224.0
3	24	Milo/salt	5,511.0	4.4	23.6	36.8	220.0
4	24	Alfalfa	1,142.0	23.8	26.2	204.1	220.0
CORRELATIONS OF THE SHIFT FROM ROUGHAGE TO CONCENTRATES							
5	10	Protein-bound hexosamine with the increasing concentrates, +0.53				Nitrogen concentration with the increasing concentrates, −0.15	

RESULTS

During the period of feeding of the milo ration, 1 lamb abruptly ceased urinating and, after euthanasia, was found to have the urethra obstructed with calculi.

The total daily urine volumes and the averages of the urine analyses for the first four feeding periods of 24 days each are shown (table 2). Both the concentration per unit of volume and the total 24-hour excretion are given for the protein-bound hexosamine and the nitrogen.

The correlations of the level of concentrate intake with the output of protein-bound hexosamine and with the output of protein nitrogen are shown (table 2).

A statistical analysis of the urinary output data during the first four periods, presented in part 1 of table 2, is shown (table 3). The method of using single degrees of freedom to make orthogonal comparisons was used in preparing this table, and three comparisons were made of each set of data for the four dietary treatments.

The comparisons made for the daily urine volumes, the concentration of protein-bound hexosamine per unit of volume, and the concentration of protein nitrogen per unit of volume were, first, a comparison of the effect of the 10 per cent NaCl ration with the combined average of the other three rations to determine the magnitude and significance of the changes induced by the force feeding of salt. A second comparison was made between the alfalfa ration and the combined average of the corn and milo rations to determine the magnitude and significance of the use of roughages as opposed to concentrates. The third comparison was between the outputs on the two types of concentrates, corn versus milo.

TABLE 3—Orthogonal Comparisons of Data Presented in Table 2

	Quantitative effect	"p"
TOTAL URINE VOLUME		
Excretion per 24 hours (ml.)		
C-1 Aver. vol. on milo/salt ration with aver. vol. of other three rations	+4,705.0 ml.	<.001
C-2 Aver. vol. on alfalfa ration with aver. vol. of milo and corn rations	+505.0 ml.	<.001
C-3 Aver. vol. on milo ration with aver. vol. of corn rations	.....	n.s.
PROTEIN-BOUND HEXOSAMINE IN URINE		
Concentration per unit of volume (μg./ml.)		
C-4 Aver. concen. on milo/salt with aver. concen. on other three rations	−28.7 μg./ml.	<.001
C-5 Aver. concen. on alfalfa with aver. concen. on corn and milo rations	−14.9 μg./ml.	<.001
C-6 Aver. concen. on milo with aver. concen. on corn rations	+5.1 μg./ml.	<.01
PROTEIN-BOUND HEXOSAMINE IN URINE		
Total excretion per 24 hours (mg./24 hrs.)		
C-7 Aver. excretion on corn ration with the other three rations	−4.0 mg./24 hr.	<.01
C-8 Aver. excretion on alfalfa ration with milo and milo/salt rations	.....	n.s.
C-9 Aver. excretion on milo with milo/salt rations	.....	n.s.
PROTEIN NITROGEN IN URINE		
Concentration per unit of volume (μg./ml.)		
C-10 Aver. concen. on milo/salt with aver. concen. on other three rations	−269.2 μg./ml.	<.001
C-11 Aver. concen. on alfalfa with aver. concen. on corn and milo rations	−153.0 μg./ml.	<.001
C-12 Aver. concen. on milo with aver. concen. on corn rations	.....	n.s.
TOTAL EXCRETION PER 24 HOURS		
C-13 There were no significant differences among these averages	.....	n.s.

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The comparisons made for the total 24-hour outputs of hexosamine and nitrogen were different. The first comparison was between the average output on corn with the combined average outputs of the other three rations to determine whether the total daily excretion on corn was significantly lower. The second comparison was between the alfalfa and the two milo rations and, finally, a comparison between the two rations containing milo was made.

DISCUSSION

If it is assumed that phosphotungstic acid precipitates the mucoproteins that occur in the urine, that the hexosamine distinguishes the mucopolysaccharide moiety, and that the nitrogen is largely from the protein moiety, it is apparent from the correlations observed that the ration affects the amounts of the mucopolysaccharide appearing in the urine but has no effect on the quantity of protein. Changes in the amount of mucopolysaccharide can be explained by the changes in concentration in the plasma and, hence, in the glomerular filtrate, assuming that the material is not reabsorbed by the tubules.

The problem of the source of urinary mucopolysaccharides has been discussed by Kerby [7] and, though there is little evidence regarding the amounts of mucopolysaccharides in the plasma, only minute amounts would be needed to supply the amount found in the urine. The effects of tubular function on the amounts of mucopolysaccharides have been studied, and Piper [9] has suggested the possibility of both tubular excretion and glomerular filtration from studies of the fate of injected heparin. This presupposes that the molecular size is in the order of that of inulin, and there is some evidence that this may be true if the material is a chrondroitin sulfate or similar molecule.[8] Several sources of such materials are apparent.

In an earlier trial with steers,[13] it was shown that a high level of calcium in the ration increased the protein-bound hexosamine, probably through absorption of the calcium on the bone tissue, accompanied by an increased rate of turnover of the matrix of this tissue, since Dziewiatowski [5] has reported that absorption of calcium increases the turnover of the bone matrix. Other sources are the ground substances of connective tissue, and an increased turnover of these induced by stimulation of the growth rate appears to occur with a high-concentrate ration.

The probable importance of the turnover rate of the supporting tissues is emphasized by the increased probability of urinary calculi in lambs which have been implanted with diethylstilbestrol to increase growth rate.[14] It seems probable that the greatest predisposition to calculosis occurs during periods when the animal is growing rapidly, as when it is being fed for market, and that the imposition of an additional stress at this time, such as an abrupt shift in the weather, may result in the changes necessary to bring about the deposition of mineral on the mucoproteins present in the urine.

SUMMARY

A study was made of the effects of certain rations, previously shown to promote or inhibit the incidence of clinical urolithiasis, on the patterns of excretion of urinary mucoproteins.

The concentrations of the urinary mucoproteins were found to be inversely proportional to the levels of minerals or ash in the ration, but under the conditions of this trial the ash content of the ration did not affect the total amounts of these materials excreted per day.

Evidence was presented that the concentration of the mucopolysaccharide portion of the mucoproteins of the urine was correlated directly to the level of concentrates in the ration, and the hypothesis was advanced that the correlation was the result of the increased tissue turnover rate induced by an increased growth rate.

Evidence was also presented that the level of the protein fraction of the mucoprotein in the urine was not related to the amounts of concentrate fed. This suggests that the two portions of the complex are derived from different sources.

References

[1] Black, W. H., Ellis, N. R., Jones, J. M., and Keating, F. E.: The Relation Between Urinary Calculi and the Use of Grain Sorghums in Steer Fattening Rations in the Southern Great Plains. U.S.D.A. Tech. Bull. 945, (1942): 14.

[2] Boyce, W. H., Garvey, F. K., and Norfleet, C. M.: Biocolloids in Health and Disease. I. Electrophoretic Studies at pH 8.6 and 4.5 of Those Components Soluble in Molar Sodium Chloride. J. Clin. Invest., *33*, (1954): 1287–1297.

[3] Boyce, W. H., and Swanson, M.: Biocolloids in Health and Disease. II. Electrophoretic and Biochemical Studies of a Mucoprotein Insoluble in Molar Sodium Chloride. J. Clin. Invest., *34*, (1955): 1581–1589.

[4] Boyce, W. H., and Sulin, N. M.: Biocolloids in Health and in Calculous Disease. III. The Mucoprotein Matrix of Urinary Calculi. J. Clin. Invest., *35*, (1956): 1067–1079.

[5] Dziewiatkowski, D. D.: Radioautographic Studies of Sulfate-Sulfur^{35} Metabolism in Articular Cartilage. J. Expt. Med., *95*, (1952): 489–495.

[6] Howard, J. E.: Clinical and Laboratory Research Concerning the Mechanisms of Formation Calculous Disease of the Kidney. J. Urol., (1954): 999–1008.

[7] Kerby, Grace P.: The Excretion of Glucuronic Acid and Acid Mucopolysaccharides in Normal Human Urine. J. Clin. Invest., *33*, (1954): 1168–1174.

[8] Mathews, M. B., and Dorfman, A.: The Molecular Weight and Viscosity of Chondroitin Sulfuric Acid. Arch. Biochem., *42*, (1953): 41–48.

[9] Piper, J.: The Fate of Heparin in Rabbits After Intravenous Injection. Acta Pharmacol. et toxicol., *3*, (1947): 737–741.

[10] Randall, A.: The Origin and Growth of Urinary Calculi. Ann. Surg., *105*, (1937): 1009–1027.

[11] Rubin, P. S., and Howard, J. E.: Histochemical Studies of the Role of Acid Mucopolysaccharides in Calcifiability and Calcification. Tr. Macy Conf. Metab. Interrelat., *2*, (1950): 155–166.

[12] Tobiska, J. W., Douglas, E., Vail, C. E., and Morris, M.: Nutritional Characteristics of Some Mountain Meadow Hay Plants of Colorado. Colorado Agric. Exper. Sta. Tech. Bull. 21, (1937): 1–23.

[13] Udall, R. H., Deem, A. W., and Maag, D. D.: Studies on Urolithiasis. I. Experimental Production in Steers Associated with Feeding. Am. J. Vet. Res., *19*, (Oct., 1957): 825–829.

[14] Udall, R. H., and Jensen, R.: Studies on Urolithiasis. II. The Occurrence in Feedlot Lambs Following Implantation with Diethylstilbestrol. J.A.V.M.A., *133*, (Oct. 15, 1958): 514–516.

[15] Udall, R. H.: Studies on Urolithiasis. III. The Control by Force Feeding Sodium Chloride. Am. J. Vet. Res., *20*, (May, 1959): 423–425.

[16] Winzler, R. V., Devor, A. W., Mehl, J. W., and Smyth, I. W.: Studies on the Mucoprotein of Human Plasma. I. Determination and Isolation. J. Clin. Invest., *27*, (1948): 609–615.

Correction—Sulfaquinoxaline in Dairy Cattle

In the article, "Studies on the Oral Administration of Sulfaquinoxaline in Dairy Cattle—Pharmacology and Pathology," by G. E. Brightenback, F. V. Washko, and O. H. Siegmund, in the October, 1958 issue of the *American Journal of Veterinary Research* (p. 794), the last paragraph of the Discussion, beginning with the sentence starting on line six, should read "*Unpublished reports*[28] *attest to the efficacy of this drug. . .*" Reference 28, not included in the article, should read "[28] Stowe, C. M., Pallesen, D., and Hartman, W., University of Minnesota, St. Paul: Unpublished data, 1958.

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Acidosis and Plasma Glucose in Sheep Given Butyrate

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HYPERKETONEMIA and hypoglycemia are associated with metabolic disease in cattle (bovine ketosis) and sheep (ovine pregnancy toxemia).

This has promoted research on the effects on blood concentrations of ketone bodies and glucose which follow the administration of the volatile fatty acids produced by ruminal fermentation.[5, 14, 16, 18, 19, 25, 28, 29] Propionate always raises the blood sugar and depresses the ketone body level,[5, 14, 16, 25, 28] acetate has slight effects, while butyrate always raises the ketone concentration, but the direction of change in glucose concentration is variable and, until recently,[18] was thought to be unpredictable.[5, 14, 16]

The common nutritional approach to ketosis based on the classification of nutrient substances as ketogenic or antiketogenic[20] and the identity of antiketogenic with ketolytic and glycogenic substances[21] has made the erratic glycogenic effect of butyrate difficult to understand in the face of the consistent ketogenic effect.[5, 8, 18] Theories of ruminant ketosis which emphasize the glycogenicity of propionate and the ketogenicity of butyrate have minimized or ignored the erratic glycogenicity of the latter.[2, 9, 15] It can be calculated that if all the propionate produced in the rumen was transformed to glucose it would provide only 40 Gm. per day in the sheep and 400 Gm. per day in the cow. These amounts are inadequate to provide for demands on blood glucose.[1, 5, 6, 20] Thus the variables which condition the glycogenicity of butyrate assume great importance for, when this transforming mechanism is not operating, the animal is more likely to become deficient in blood sugar. This would be especially dangerous if the animal faced a concomitant drain of hexose for milk lactose or fetal fructose.[1, 20] Hypoglycemia has been closely related to the development of the syndrome in ovine pregnancy toxemia[21] and, less critically, in bovine ketosis.[32]

A variable which affects this two-way directional effect of butyrate on blood sugar was demonstrated by Kronfeld.[19] The blood sugar level fell during butyrate infusiion in sheep which had been previously loaded with glucose by intravenous infusion, but it rose when butyrate was given intravenously to sheep which had been starved for four days. The change in blood glucose correlated with the initial level ($r = -0.848$) at a highly significant level ($P < 0.001$) for 15 observations.[19] Annison *et al.*[3] have not accepted this as evidence for a glycogenic effect of butyrate, preferring to consider it a pharmacological effect since the butyrate was given intravenously and it is not normally present in peripheral blood. They gave sodium butyrate intraruminally to fasted sheep, avoiding the possible pharmacological effect, and still observed a rise in blood sugar.

A comparison of the result obtained by Annison *et al.*[3] with that obtained by Armstrong and Blaxter,[4] who infused butyric acid into the rumens of fasted sheep and observed a fall in blood sugar has led to the postulation of another variable, acidosis, affecting the glycogenic action of butyrate. This has been tested by giving sodium butyrate and butyric acid intravenously and intraruminally to sheep and comparing the effects on blood sugar and CO_2-combining power.

EXPERIMENTAL PROCEDURE AND RESULTS

The procedures followed were those of Kronfeld[19] for intravenous infusion and Armstrong and Blaxter[4] for intraruminal infusion. The sheep were crossbred adult females. Plasma glucose concentration was estimated photometrically with ferricyanide on a $ZnSO_4$-NaOH filtrate.[12, 33] Plasma CO_2-combining power was estimated volumetrically.[30] The sole variation was the use by each route of butyric acid in saline solution (pH 2.0) and the same amount of butyric acid titrated to pH 7.4 with NaOH.

In four experiments the sheep became comatose and dyspneic, and their plasma glucose levels rose to between 120 and 150 mg. We agree with Jarrett *et al.*[14] that such a marked increase in plasma glucose accompanied by signs of intoxication is probably part of the response to the toxic action of butyrate rather than a sign of glycogenicity. In five other experiments (graphs 1, 2), the sheep became only slightly dull and unresponsive during the infusion period but otherwise showed no signs of intoxication, and we consider the rise in plasma glucose level to 60 and 70 mg. in these experiments an indication of glycogenicity. It is hoped to continue

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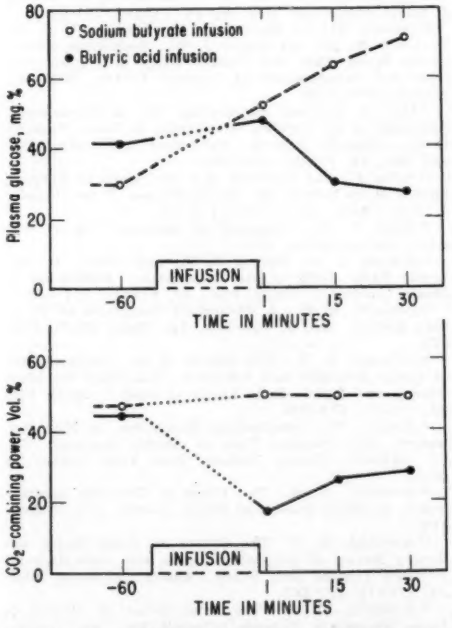
this work, using tracer techniques which would obviate the problem of butyrate toxicity and which would provide a different criterion of glycogenicity.

The increase in plasma glucose concentration which followed the administration of sodium butyrate intravenously (graph 1) and intraruminally (graph 2) was not accompanied by acidosis. A fall in the CO_2-combining power of plasma did occur, however, after the administration of butyric acid by either route. It was associated with a fall in plasma glucose. These observations confirm those previously reported. Kronfeld [18] found a rise in glucose after sodium butyrate was infused intravenously into fasted sheep. Annison *et al.*[3] observed a rise in blood sugar following the intraruminal administration of sodium butyrate, while Armstrong and Blaxter [4] reported a fall in blood sugar during the intraruminal infusion of butyric acid.

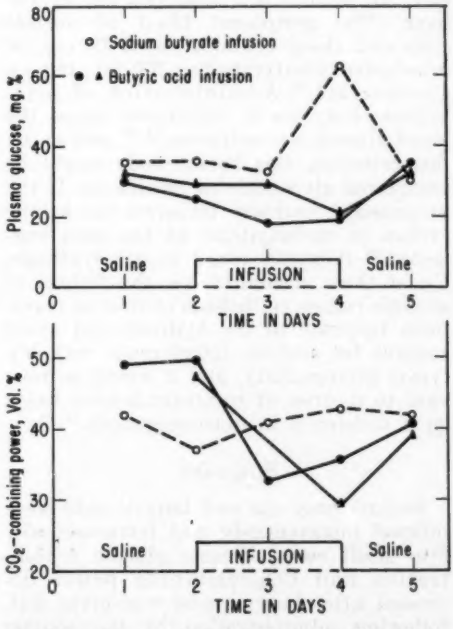
Discussion

The results are consistent with the hypothesis that acidosis blocks the glycogenic effect of butyrate. Acidosis also diminishes the energetic efficiency of volatile fatty acid and glucose metabolism in sheep.[4] Both effects would tend to lower the plasma level, and so have interesting implications in ruminant ketosis.

Obese ewes are especially susceptible to pregnancy toxemia.[7] Sampson [27] has long held that ruminant ketosis is dependent on depletion of liver glycogen, but this opinion may require requalification, since recent work has shown that fasting ketosis can occur in the presence of undepleted liver glycogen [22] and undepleted oxalacetate.[34] Fasting ketosis [7] due to fat mobilization under the influence of pituitary growth hormone [26] might be primary in obese ewes, leading to acidosis and then to hypoglycemia and glycogen depletion along the lines proposed. Again, impaired renal function, although terminal when pregnant ewes in ordinary condition are fasted,[23] occurs early and in association with acidosis when obese sheep are starved [13] and could be responsible for the development of subsequent hypoglycemia. The onset of clinical signs parallels the development of hypoglycemia in pregnancy toxemia.[21] The



Graph 1—Intravenous infusion of sodium butyrate and butyric acid on plasma glucose and CO_2-combining power in fasted sheep.



Graph 2—Intraruminal infusion of sodium butyrate and butyric acid on plasma glucose and CO_2-combining power in fasted sheep.

sequence of ketosis-acidosis-cellular glucose insufficiency-clinical signs in the fasted, fat ewe contrasts with that of cellular glucose insufficiency-ketosis-acidosis-clinical signs which are characteristic of diabetes.

The relative magnitude of different pathways by which butyrate carbon proceeds to glycogen and glucose might also be influenced by acidosis. Radioactive butyrate given intravenously has labeled amino acids recovered from milk casein in a pattern consistent with incorporation via acetyl-CoA entry into the Krebs cycle, and transfer of butyrate carbon to plasma glucose was also considered to follow this route.[17] Incorporation via acetyl-CoA in this way would not result in a net increase in the number of carbon atoms in the carbohydrate pool because, for every two carbons entering the Krebs cycle as acetyl-CoA, another two would be lost as carbon dioxide; that is, there would be no net synthesis of glucose. However, butyrate is not normally present in peripheral blood;[3,10] most of it is transformed to acetoacetate and *beta*-hydroxy-butyrate by the rumen epithelium during the process of absorption,[3,24] the remaining butyrate and the acetoacetate disappearing at the liver. The peripheral blood of normal cows and sheep contains about 12 mg. of *beta*-hydroxy-butyrate per 100 ml., but no acetoacetate.[35] Administration of *beta*-hydroxy-butyrate to ruminants raises the blood glucose concentration,[19,20] and so, by this criterion, this ketone body might be considered glycogenic in ruminants. If the propanediol pathway transfers the ketone carbon to carbohydrate as has been suggested,[19] it would result in net synthesis. Under these circumstances, the failure of acidotic rumen epithelium or liver to transform butyrate to the hydroxy-acid could account for acidotic interference with butyrate glycogenicity, and it would be relevant to theories of ruminant ketosis based on a deficiency in gluconeogenesis.[11,15]

SUMMARY

Sodium butyrate and butyric acid were infused intravenously and intraruminally into adult ewes. Plasma glucose concentration and CO_2-combining power decreased after butyric acid was given but, following administration of the sodium salt, there was no acidosis and the plasma glucose level was increased. It was concluded that acidosis interfered with the glycogenic effect of butyrate. Implications of this were discussed with reference to ruminant ketosis.

References

[1] Adler, J. H., and Dye, J. A.: On the Mechanism of Ketogenesis in Ruminants. Cornell Vet., *46*, (1955): 58–63.

[2] Adler, J. H., Roberts, S. J., and Dye, J. A.: Further Observations on Silage as a Possible Etiological Factor in Bovine Ketosis. Am. J. Vet. Res., *19*, (April, 1958): 314–318.

[3] Annison, E. F., Hill, K. J., and Lewis, D.: Studies on the Portal Blood of Sheep. II. Absorption of Volatile Fatty Acids from the Rumen of Sheep. Biochem. J., *66*, (1957): 592–599.

[4] Armstrong, D. G., and Blaxter, K. L.: The Heat Increment of Steam-Volatile Fatty Acids in Fasting Sheep. Brit. J. Nutr., *11*, (1957): 247–267.

[5] Baxter, C., Kleiber, M., and Black, A. L.: Glucose Metabolism in the Lactating Dairy Cow. Biochim. et biophys. acta, *17*, (1955): 354–361.

[6] Carroll, E. J., and Hungate, R. E.: The Magnitude of the Microbial Fermentation in the Bovine Rumen. Appl. Microbiol., *2*, (1954): 205–214.

[7] Clark, R.: Domsietke or Pregnancy Disease in Sheep. IV. The Effect of Obesity on the Reaction of Sheep to a Sudden Reduction in Diet. Onderstepoort J. Vet. Res., *18*, (1943): 279–288.

[8] Clark, R., and Malan, J. R.: Alterations in the Blood Sugar and Ketone Levels Caused by Dosing Acetate, Proprionate and Butyrate into the Rumen of the Sheep. Onderstepoort J. Vet. Res., *27*, (1956): 101–109.

[9] Clark, R., and Weiss, K. E.: Carbohydrate Metabolism in Ruminants with Special Reference to Ketosis. J. South Africa Vet. Med. A., *26*, (1955): 217–220.

[10] Conrad, H. R., Smith, H. R., Vandersall, J. H., Pounden, W. H., and Hibbs, J. W.: Estimating Gastro-splenic Blood Flow and Volatile Fatty Acid Absorption from the Forestomachs of Calves. J. Dai. Sci., *41*, (1958): 1094–1099.

[11] Dye, J. A., and McCandless, E.: A Physiological Approach to the Problem of Ketosis. I. Basic Features of the Metabolic Processes Concerned in Ketosis. Cornell Vet., *38*, (1948): 331–340.

[12] Folin, C., and Malmros, H.: An Improved Form of Folin's Micro-Method for Blood Glucose Determination. J. Biol. Chem., *83*, (1929): 115–120.

[13] Holm, L. W.; University of California, Davis: Personal communication, 1958.

[14] Jarrett, I. G., Potter, B. J., and Filsell, O. H.: Lower Fatty Acids in the Intermediary Metabolism of Sheep. Austral. J. Exptl. Biol., *30*, (1952): 311–318.

[15] Johnson, R. B.: A Theoretical Discussion of Ruminant Ketosis. Am. J. Vet. Res., *14*, (July, 1953): 366–375.

[16] Johnson, R. B.: The Effects of the Administration of Acetic, Propionic and *n*-Butyric Acids Upon the Blood Glucose and Ketone Body Levels of Goats. Cornell Vet., *45*, (1955): 273–290.

[17] Kleiber, M.: Intermediary Metabolism in Milk Formation. In "Peaceful Uses of Atomic Energy." Vol. 2. UNESCO, United Nations, New York (1958): In press.

[18] Kronfeld, D. S.: The Effect of Butyrate Administration on Blood Glucose in Sheep. Nature, *178*, (1956): 1290.

[19] Kronfeld, D. S.: The Effects on Blood Sugar and Ketone Bodies of Butyrate, Acetate and *Beta*-Hydroxy-Butyrate Infused Into Sheep. Austral. J. Exptl. Biol., *35*, (1957): 257–266.

[20] Kronfeld, D. S.: The Fetal Drain of Hexose in Ovine Pregnancy Toxemia. Cornell Vet., *48*, (1958): 394–404.

[21] McClymont, G. L., and Setchell, B. P.: Ovine Pregnancy Toxemia. IV. Insulin Induced Hypoglycemic En-

XUM

cephalopathy in the Sheep, and Its Implications as Regards Pathogenesis of the Disease. Austral. Vet. J., *32*, (1956): 97–109.

[22] Mayes, P. A.: Level of Liver Glycogen in Ketosis. Nature, *182*, (1958): 324–325.

[23] Parry, H. B., and Taylor, W. H.: Renal Function in Sheep During Normal and Toxemic Pregnancy. J. Physiol., *131*, (1956): 383–392.

[24] Pennington, R. J.: The Metabolism of Short-Chain Fatty Acids in the Sheep. I. Fatty Acid Utilization and Ketone Body Production by Rumen Epithelium and Other Tissues. Biochem. J., *51*, (1952): 251–258.

[25] Potter, B. J.: Relief of Hypoglycemic Convulsions with Butyric Acid. Nature, *170*, (1952): 541.

[26] Russell, J. A.: Effects of Growth Hormone on Protein and Carbohydrate Metabolism. Am. J. Clin. Nutr., *5*, (1957): 404–416.

[27] Sampson, J.: Ketosis in Domestic Animals. Illinois Agric. Exper. Sta. Bull. 524, (1947): 407–470.

[28] Schultz, L. H., Smith, V. R., and Lardy, H. A.: The Effect of the Administration of Various Fatty Acids on the Blood Ketone Levels of Ruminants. J. Dai. Sci., *32*, (1949): 817–822.

[29] Schultz, L. H., and Smith, V. R.: Experimental Alteration of the Sugar and Ketone Levels of Ruminants in Relation to Ketosis. J. Dai. Sci., *34*, (1951): 1191–1200.

[30] Shaffer, P. A.: Antiketogenesis. I. An *in Vitro* Analogy. J. Biol. Chem., *47*, (1921): 433–448.

[31] Shapiro, I.: Studies on Ketosis. V. The Comparative Glycogenic and Ketolytic Action of Glucose and Some Carbohydrate Intermediates. J. Biol. Chem., *108*, (1935): 373–387.

[32] Shaw, J. C.: Studies on Ketosis in Dairy Cattle. V. The Development of Ketosis. J. Dai. Sci., *26*, (1943): 1079–1090.

[33] Somogyi, M.: A Method for the Preparation of Blood Filtrates for the Determination of Blood Sugar. J. Biol. Chem., *86*, (1930): 655–663.

[34] Tapley, D. F., and Kalnitsky, G.: A Sensitive Method for Estimation of Oxaloacetate. Fed. Proc., *17*, (1958): 320.

[35] Thin, C., and Robertson, A.: Biochemical Aspects of Ruminant Ketosis. J. Comp. Path., *63*, (1953): 184–194.

[36] VanSlyke, D. D., and Cullen, G. E.: Studies of Acidosis. I. The Bicarbonate Concentration of the Blood Plasma, Its Significance and Its Determination as a Measure of Acidosis. J. Biol. Chem., *30*, (1917): 289–346.

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The Effects of Freezing and Storage at −20 C. on Serum Protein Patterns of Sheep as Determined by Paper Electrophoresis

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PAPER ELECTROPHORESIS provides a convenient means of studying various changes in serum protein components. Several investigations [1,4,7,10,13] have demonstrated the usefulness of serum electrophoresis as an aid to diagnosis in various pathological conditions. This procedure is also a useful research tool in studying the biochemical and serological aspects of certain disease problems.

In many instances, storage of serum prior to study is convenient and often necessary. Moore *et al.*[9] reported that both freezing and lyophilization cause changes in the electrophoretic patterns in human serums. Variations in electrophoretic patterns of dog serums were reported by Goldwater and Entenman [6] as a result of different types of treatment. Mathews and Buthala [8] reported that repeated freezing and thawing of swine serum did not alter the electrophoretic patterns of serum protein. Rutqvist [11] stated that no significant changes in the amounts of protein were observed in swine serums, even after prolonged storage at −60 C. Campbell [3] stated that changes in the electrophoretic patterns were not observed in serums stored at −20 C. for 6 months.

Serum components for various species of animals differ.[3,5] It would seem reasonable to suspect that changes which occur in serums of one species may not necessarily be constant with serums of another species under different types of treatment. This investigation was designed to detect possible changes which may occur in electrophoretic patterns of sheep serums as a result of freezing and storage of serums at −20 C. for 9 months.

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* Spinco Model R Paper Electrophoresis Cell, Spinco Duostat, and Spinco Model RA Analytrol, manufactured by Beckman Instruments, Inc., Spinco Division, Belmont, Calif.

MATERIALS AND METHODS

Tests were carried out with a paper electrophoresis cell.* After 10 μl. of serum were placed on each filter paper strip, the strips were placed on racks and wet down with a barbital buffer consisting of 1.84 Gm. of barbital and 10.3 Gm. of sodium barbital in 1 liter of distilled water, with a pH of 8.6. A duostat power source* supplied 15 ma. of direct current, which was used for six hours at room temperature.

The strips were dyed in bromphenol blue, using the methods described by Block *et al.*[2] Dried strips were analyzed in an integrating densitometer* using a slit width of 1.5 mm. Percentages of color content of the various serum protein fractions were calculated from this reading.

Serum samples were collected from 8 presumably normal lambs, 4 to 8 months old. A blood sample, taken from each lamb in the afternoon, was allowed to clot and separate overnight at room temperature. The next day, the serum samples were collected and clarified by centrifugation and divided into ten aliquots containing 3 ml. each. One aliquot was immediately placed on the electrophoresis cell without freezing or storage (day 0). The remaining nine aliquots were frozen and stored at −20 C. until tested. One aliquot of each serum sample was thawed and tested in duplicate at approximately one-month intervals for nine months following freezing (table 1). The test conducted in the period between 91 and 153 days was discarded, due to an inadvertent change in the technique which was not detected until the serums had been discarded.

A second group was composed of 16 presumably normal lambs, 8 to 12 months old, which were used as a source of serum in determining the effects of freezing on electrophoretic patterns. Two blood samples were taken from each lamb. Serum from the first sample (group 1) was collected and clarified by centrifugation, and placed on the electrophoresis cell about four hours after the blood samples were taken. The second blood sample was allowed to stand for seven hours at room temperature, and overnight at 5 C. before serum was collected. At this time the serum was removed, clarified by centrifugation, and divided into three aliquots containing 3 ml. each (group 2, 3, and 4). In group 2, the 16 serum samples were placed on the electrophoresis cells approximately 26 hours after blood samples were drawn and prior to freezing. In group 3, the 16 serum samples were frozen

TABLE 1—Effects of Storage at −20 C. on Electrophoretic Patterns of Sheep Serums

Days of storage	Albumin (%)	*Alpha*-1 globulin (%)	*Alpha*-2 globulin (%)	*Beta* globulin (%)	*Gamma* globulin (%)
0	46.1	6.3	16.6	6.1	25.0
32	49.4	5.6	16.1	6.1	22.8
61	48.6	5.7	15.9	6.7	23.2
91	48.0	5.6	17.4	7.0	21.9
153	48.0	5.7	15.6	6.9	23.7
182	46.1	5.9	16.6	7.2	24.1
214	45.4	5.6	18.1	7.6	23.0
245	49.4	5.0	15.5	7.4	22.6
273	47.4	4.9	17.1	8.4*	22.2
Regression coefficient	−0.006923	−0.002524	+0.002345	+0.00699	−0.000460
Possibility of error	N.S.†	N.S.	N.S.	<0.005	N.S.

Each percentage expressed represents the average of 16 tests on a total of 8 animals. Regression analysis was calculated starting with 32 days' storage.

* Significantly greater than 6.1 per cent.

† N.S. = not significant.

24 hours after blood samples were taken by placing them in −20 C. storage. They were thawed and tested by electrophoresis approximately 48 hours after the blood sample had been collected. In group 4, the 16 serum samples were quickly frozen, 24 hours after the blood samples were drawn, by placing tubes containing serum in dry ice and alcohol, having a temperature of −70 C. They were stored at −20 C. until tested, approximately 72 hours after collection of blood samples.

Uniform methods, as previously described, were used in making all electrophoretic serum protein separations. The protein fractions separated and were recorded in the following order, beginning from the point of origin on the paper strip: *gamma* globulin, *beta* globulin, *alpha*-2 globulin, *alpha*-1 globulin, and albumin.

In order to determine if changes occurring in serum electrophoretic patterns were the result of storage at −20 C. (table 1), a regression analysis[12] was made. Analysis of variance, single classification, was also made in the one case where a significant regression coefficient was obtained. The data presented on the effects of freezing on serum fractions (table 2) were submitted to an analysis of variance, single classification.

TABLE 2—Effects of Freezing on Electrophoretic Patterns of Sheep Serum

Group No.	Albumin (%)	*Alpha*-1 globulin (%)	*Alpha*-2 globulin (%)	*Beta* globulin (%)	*Gamma* globulin (%)
1	50.4	5.0	14.8	5.8	24.0
2	49.2	5.1	14.8	6.3	24.6
3	49.8	4.6	15.5	7.0	23.6
4	52.4*	4.2	13.8	6.0	23.6

Each value represents the average of 16 determinations on 16 serums.

Group 1, serum was tested before freezing, four hours after blood was collected; group 2, serum was tested before freezing, 26 hours after blood was collected; group 3, serum was frozen at −20 C., held at this temperature 24 hours, and tested 48 hours after blood was collected; and group 4, serum was quick frozen at −70 C., held at −20 C. 48 hours, and tested 72 hours after blood sample had been drawn.

* Albumin in group 4 is significantly higher.

Results

The effects of storage at −20 C. on serum electrophoretic patterns of sheep serums are tabulated (table 1). There was not a significant correlation between the storage time at −20 C. and recorded differences in percentage values of albumin, *alpha*-1 globulin, *alpha*-2 globulin, or *gamma* globulin fractions in sheep serum. As determined by an analysis of variance and Tukey's method of determining significant differences,[12] a highly significant, although small, regression coefficient did occur in the *beta* globulin fraction, indicating that prolonged storage does affect this serum fraction. The differences of 8.4 per cent at 273 days of storage were significantly different from the values of 6.1 per cent obtained on days 0 and 32. The differences between 7.4 per cent obtained on day 245 or 7.6 per cent on day 214, and the value of 6.1 per cent on days 0 and 32, did not reach significance.

The effects of freezing on serum electrophoretic patterns are given (table 2). No apparent differences in electrophoretic patterns developed as a result of the 3 different treatments represented in group 1, 2, and 3. Group 4, in which the serum was quickly frozen in dry ice and alcohol, showed an increase in the albumin fraction which proved to be significant. This difference reached significance when compared with each of the other three groups.

Discussion

The results obtained in this study of the effects of freezing and storage on serum electrophoretic patterns, where repeated determinations were made on the same serums, indicate that this test is sensitive to slight changes in the uniform method.

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It appears that serum could be safely stored without significant changes occurring in the electrophoretic patterns of the albumin, *alpha*-1 globulin, *alpha*-2 globulin, and *gamma* globulin fractions, but that if storage of over eight months is contemplated, storage changes in the *beta* globulin fraction should be anticipated. Although it is small, the regression coefficient given (table 1) for the *beta* fraction has the possibility of error of <0.005.

Freezing serum samples at −20 C. appears to be preferable to quick freezing in dry ice and alcohol when results comparable to those obtained in fresh unfrozen samples are desired. Holding serum overnight in a refrigerator failed to produce any differences in electrophoretic patterns when compared to those patterns obtained on fresh serum.

SUMMARY

No significant changes in the electrophoretic patterns of sheep serums occurred in the albumin, *alpha*-1 globulin, *alpha*-2 globulin, and *gamma* globulin fractions as a result of nine months' storage at −20 C. A small, but significant, increase occurred in the *beta* globulin fraction, apparently due to storage.

There was no significant change in the electrophoretic patterns as a result of freezing at −20 C., although quick freezing at −70 C. produced a small but significant increase in the albumin fraction.

References

[1] Banerjee, S., and Chatterjee, K. P.: Different Fractions of Plasma Proteins in Some Infectious Diseases. Proc. Soc. Exptl. Biol. & Med., *94*, (1957): 474.

[2] Block, R. J., Durrum, E. L., and Zweig, G.: A Manual of Paper Chromatography and Paper Electrophoresis. Academic Press, New York, N.Y., 1955.

[3] Campbell, E. A.: The Use of Paper Electrophoresis as an Aid to Diagnosis. J. Comp. Path. & Therap., *67*, (1957): 345.

[4] Chabaud, M. A., Serie, C., and Ondral, L.: Electrophorèse et diagnostic de la rage. Ann. Inst. Pasteur., *88*, (1955): 420.

[5] Dukes, H. H.: The Physiology of Domestic Animals. 7th ed., Comstock Publishing Associates, Ithaca, N.Y., 1955.

[6] Goldwater, W. H., and Entenman, C.: Variations in Paper Electrophoretic Patterns of Dog Sera and Plasmas Prepared by Different Technics. Proc. Soc. Exptl. Biol. & Med., *94*, (1957): 486.

[7] Hussien, R., Moustafa, E. M., and Salam, H.: The Use of Electrophoretic Pattern of Blood Serum Proteins in the Diagnosis of Tuberculosis in Buffaloes. Brit. Vet. J., *113*, (1957): 504.

[8] Mathews, J., and Buthala, D. A.: Effect of Repeated Freezing and Thawing on the Electrophoretic Pattern of Swine Serum. Am. J. Vet. Res., *17*, (July, 1956): 485.

[9] Moore, D. H., Roberts, J. B., Costello, M., and Schonberger, T. W.: Factors Influencing the Electrophoretic Analysis of Human Serum. J. Biol. Chem., *180*, (1949): 1147.

[10] Rooney, J. R.: Paper Electrophoresis of Bovine Serum. Am. J. Vet. Res., *18*, (Jan., 1957): 67–72.

[11] Rutqvist, L.: Electrophoretic Patterns of Blood Serum from Pig Fetuses and Young Pigs. Am. J. Vet. Res., *19*, (Jan., 1958): 25.

[12] Snedecor, G. W.: Statistical Methods. 5th ed. Iowa State College Press, 1956.

[13] Williams, M. W., Fitzhugh, O. G., and Cook, J. W.: Serum Protein Changes Following Feeding of Parathion to Dogs. Proc. Soc. Exptl. Biol. & Med., *96*, (1957): 539.

The Presence of Lipids in the Epithelium of the Ruminant Forestomach

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THE IMPETUS for this study was furnished by the literature on the absorption of fatty acids from the rumen, reticulum, and omasum,[2,7] the metabolism of fatty acids by the tunica mucosa of the forestomach,[17,18] and the influence of fatty acids on the development of papillae.[10]

There is no previous report of the presence of lipids in the stratum corneum of the ruminant forestomach. The latest positive reference to the histological demonstration of lipids in the tunica mucosa is in Ellenberger's "Handbuch" of 1911.[9] Even here we find only the meager information that fat droplets occur in the stratum spinosum and stratum basale. Hauser's comprehensive description of the epithelium[14] contains no mention of lipids. Barcroft *et al.*,[3] comparing the rumen epithelium to the skin, reasoned that because the rumen epithelium has no sebaceous glands, " . . . there is no greasy protection . . . " The belief that the lipids of the epidermis are derived entirely from the glands has been shown to be erroneous. Substantial quantities of fatty acids and their esters are produced by the transformation of cellular phospholipids during the process of keratinization.[15,16]

Dobson *et al.*[5] were unable to demonstrate lipids in the ovine rumen epithelium by staining with scharlach I or Nile blue sulfate. Basu *et al.*,[4] employing Fischler's method for fatty acids, obtained the inconclusive results usually reported for this technique.

MATERIALS AND METHODS

The tissue samples were taken from 10 cattle, 2 goats, and 1 sheep. These animals are listed with their identifying number, age, and diet. Some of the animals were subjected to experiments designed for other purposes, and the treatments are indicated.

41—Calf, 1 day old. Colostrum.

42—Calf, 9 weeks old. Normal diet of hay and grain.

43—Heifer, 18 months old. Normal diet. Adrenalectomy was performed on the right side five weeks before necropsy, and on the left side two weeks before necropsy. A remnant of the right adrenal, weighing 4 Gm., was not removed.

44—Goat, 3 months old. Weaned at six weeks, normal diet.

47—Goat, 3 months old. Weaned at eight weeks and restricted to an artificial diet of dextrin, dextrose, cellulose, minerals, urea, casein, lard, and vitamins A and D.

48—Calf, 7 weeks old. Normal diet.

49—Bull, 3 years old. Normal diet.

50—Bull, 3 years old. Normal diet.

51—Bull, 10 months old. Diet restricted to milk, minerals, and vitamin E.

52—Bull, 10 months old. Diet restricted to milk, minerals, and vitamin E. The removal of tissue blocks was delayed 45 minutes after death. The rumen and reticulum were filled with water and submerged for volume measurements. The omasum was only submerged.

53—Sheep, 18 months old. Normal diet.

54—Calf, 15 months old. Diet restricted to milk fed from a nipple pail, and a solution of sodium acetate administered through a rumen cannula.

55—Calf, 15 months old. Diet restricted to milk fed from a nipple pail, and a solution of sodium propionate administered through a rumen cannula.

Tissue blocks were removed as soon as possible after exsanguination from all animals except animal 52. Blocks were cut from four places in the dorsal sac of the rumen—the cranial and caudal extremities, and dorsal and ventral locations in the middle of the right wall. Blocks were also taken from the floor of the ventral sac of the rumen, from the reticulum, and from the greater curvature of the omasum. The tissues were fixed in 10 per cent formalin containing 1 per cent calcium chloride (animals 41–50) or in formalin-calcium with 1 per cent cadmium chloride added (animals 51–55). The blocks were post-chromed in 3 per cent aqueous potassium dichromate for three to seven days and imbedded in gelatin. Frozen sections were cut at a thickness of 10 μ and attached to gelatin-coated slides.[6] Staining with Sudan black B and oil red O was carried out in the usual manner,[12] except that the saturated solutions of the dyes in 70 per cent alcohol were not boiled or filtered. The sections were stained ten minutes in Sudan black B or 20 minutes in oil red O. Because tissues did not adhere to gelatin slides in 1 per cent Nile blue sulfate, they were carried through this procedure[5] as free sections. Unfixed and unstained frozen sections were examined with dark field, phase contrast, and

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polarized illumination. All preparations were mounted from water in corn syrup (Karo).

The Schultz cholesterol reaction[19] was applied to sections from animals 48, 51, and 53.

RESULTS

Stratum Corneum.—In 9 of the 13 animals examined, the stratum corneum of the rumen, reticulum, and omasum contained large numbers of lipid droplets which stained intensely with Sudan black B and oil red O (fig. 1, 2). Most of the droplets were 1 to 3 μ in diameter. They were more numerous on the tips of the papillae than elsewhere, and more numerous and more evenly distributed in young animals than in adults given a similar diet of hay and grain. The droplets were especially large (about 5 μ in diameter) in the milk-fed yearling, animal 51 (fig. 3).

The four animals in which the lipids of the stratum corneum were sparse or absent were animals 41, 43, 52, and 53. In the day-old calf (41) no substance stained with oil red O or Sudan black B was present in the stratum corneum. A control on the method was provided by well-stained fat cells in the stomach wall and by fat droplets in the lumen.

With the exception of a few coarse droplets in the reticulum, the stratum corneum of the partially adrenalectomized heifer (43) contained almost no lipid.

The distribution of stainable lipids in animal 52 was erratic. The droplets were seen in only a few papillae of the rumen and reticulum, but were more numerous in the omasum.

The sheep (53) had less lipid in the stratum corneum than the cattle or the goats. The droplets were small (fig. 4), and stained with Sudan black B and oil red O, but not with Nile blue sulfate.

Deep Layers of the Epithelium.—In most animals, droplets stained with oil red O were rare in the layers of epithelium beneath the stratum corneum. They were present between the cells and within a few isolated cells of the stratum spinosum. Occasional cells of the stratum granulosum were filled with them. In animal 49, the stratum granulosum contained many droplets, and in animal 51 they were numerous throughout the epithelium.

Lipids stained by Sudan black B and the blue base of Nile blue sulfate, but not by oil red O, were present as a finely granular material in the cytoplasm of the stratum basale of all animals, and in the stratum spinosum of all animals except the day-old calf. In the deeper, vertically elongated cells this sudanophilic material formed a supranuclear cap (fig. 5). A Golgi vesicle with a few peripheral sudanophilic droplets was often attached to the nucleus. In the more superficial horizontal cells, the sudanophilic granules formed an elliptical zone around the nucleus (fig. 6). The Sudan black B also stained the cell membranes, frequently with a beaded appearance.

The Nature of the Lipids.—Although chemical identification of the lipids present will have to await further investigation, the following results of preliminary observations are reported.

Legend for Illustration on Opposite Page

Fig. 1—Papillae from the rumen of calf 48. Lipid droplets are especially well demonstrated in the oblique section through the stratum corneum of the largest papilla. The deeper layers of the epithelium are also stained. Sudan black B stain; x 120.

Fig. 2—Papilla from the rumen of goat 44. The lipid droplets in the stratum corneum are stained, but the deeper layers of the epithelium are not. Oil red O stain; x 120.

Fig. 3—Rumen epithelium from milk-fed bull 51. The lipid droplets in the stratum corneum are large (compare with figure 6). Sudan black B stain; x 530.

Fig 4—Rumen epithelium from sheep 53. The swollen cells of the stratum corneum contain fine lipid droplets. Sudan black B stain; x 530.

Fig. 5—Rumen epithelium from goat 44. Notice the lipid droplets in the stratum corneum, the diffuse sudanophilia of the upper cells of the stratum spinosum, the cell membranes, and the supranuclear zones of lipid material in the stratum basale. The nuclei are unstained. Sudan black B stain; x 1,060.

Fig. 6—Rumen epithelium from bull 50. Coarse lipid droplets are present in the stratum corneum. Cell membranes and perinuclear zones of lipid material are shown in the stratum spinosum. Sudan black B stain; x 530.

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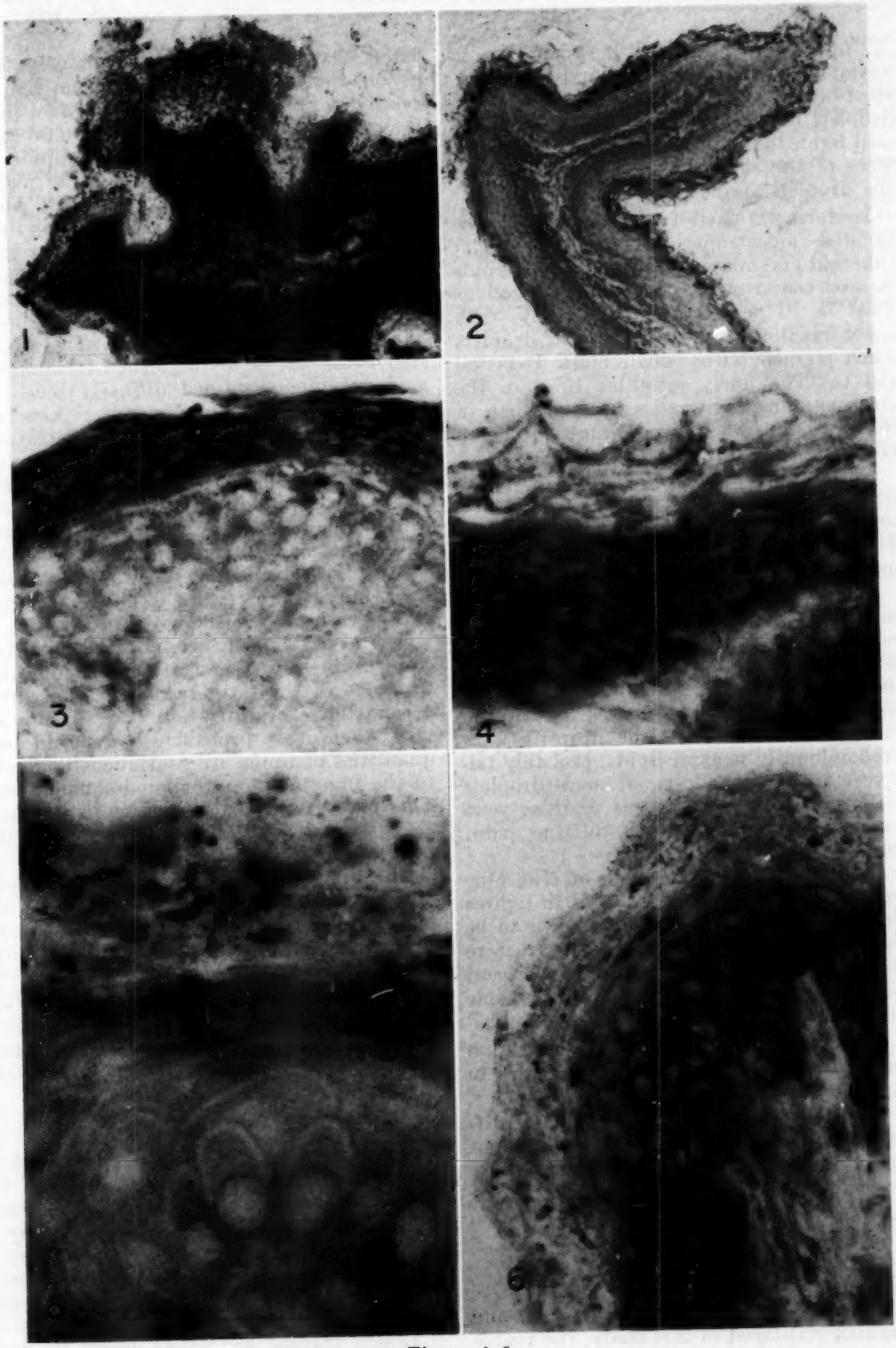

Figures 1–6

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Unfixed frozen sections of rumen mucosa examined in the bright field and phase contrast systems showed refractive colorless droplets in the stratum corneum. In polarized light, the droplets were isotropic. They were of the same size and distribution as the droplets stained with oil red O.

In formalin-fixed frozen sections a few isolated, anisotropic, elliptical, or rarely, spheroid crystals were seen. The spheroids showed the cross of polarization. Sections fixed in 10 per cent formalin only, with no chromation, revealed the lipid droplets when stained with Sudan black B or oil red O. The finely granular lipid in the stratum spinosum was less distinct than in the formalin-calcium-cadmium chromated material.

The droplets in the stratum corneum were not stained by oil red O after extraction for 24 hours in ether-alcohol at 37 C. They were also extracted by the paraffin imbedding process.

Nile blue sulfate in 1 per cent aqueous solution stained the droplets of the stratum corneum in shades ranging from pink to lavender. More droplets were stained and the color was redder when the dye solution was kept at 70 C. than at 37 C. The pink reaction indicated that the droplets were predominantly neutral lipids, probably triglycerides. The staining of more droplets at 70 C. indicated that some of them were composed of lipids with a melting point higher than 37 C.

In a 0.02 per cent solution of Nile blue sulfate the droplets were stained in lighter shades of pink. No elements shown to be lipid in Sudan black preparations were stained intensely blue with 0.02 per cent Nile blue. This indicated that the visible droplets were not composed of fatty acids.[5]

The cytoplasm of the cells of the stratum basale and spinosum was stained blue in both concentrations of Nile blue sulfate.

The Schultz cholesterol test gave a diffuse, light, blue-green reaction throughout the epithelium. The color changed rapidly to yellow brown. Myelin sheaths in the myenteric plexus gave a darker blue reaction than the epithelium.

DISCUSSION

Under the conditions of fixation and staining employed in this study, the visible droplets in the stratum corneum had physical and chemical properties which indicated that they were predominantly triglycerides, and that some of them had a relatively high melting point. The fact that they were stained with oil red O would cast doubt on the presence of phospholipids in any significant quantity,[13] and the negative results with 0.02 per cent Nile blue sulfate did not support the hypothesis that the droplets were composed of fatty acids. The short-chain, volatile, fatty acids absorbed from the ingesta are miscible with water and could not be expected to appear as discrete refractive droplets. Therefore, they could not be distinguished from other acid substances stained diffusely blue by 0.02 per cent Nile blue sulfate. Another indication that the lipids in the stratum corneum were not absorbed from the ingesta was furnished by the milk-fed bull (51). The epithelium in this animal contained much more lipid than in animals with a normally functioning rumen. It is not likely that milk fat was absorbed from the rumen. The results with the day-old calf were in agreement with this.

The available evidence indicates that the lipid droplets in the stratum corneum are products of the fatty transformation that accompanies keratinization.[1, 15, 16] If this is true, then it is probable that significant quantities of lipids are continuously added to the ingesta by epithelial desquamation. This process may be important in the biochemistry of the ruminant forestomach in health and disease.

The lipids in the deeper layers, stained by Sudan black B and the blue base of Nile blue sulfate, are probably phospholipids.[5, 13] The distribution of sudanophilic material in the cytoplasm resembles that of mitochondria. The absence of sudanophilia in the cells of the stratum spinosum of the day-old calf may be explained by the fact that these cells are still packed with fetal glycogen.[11, 12]

SUMMARY

Lipid droplets were demonstrated in the stratum corneum of the forestomach of 9 cattle, 2 goats, and 1 sheep. No lipid was stained in the stratum corneum of a day-old calf. Evidence was presented to support the hypothesis that the droplets are predominantly triglycerides produced in the normal process of keratinization.

Lipids stained by Sudan black B, but not by oil red O, were present as finely granular material in the cytoplasm of the

stratum basale of all 13 animals, and in the stratum spinosum of all except the day-old calf. Sudan black B also stained the cell membranes.

References

[1] Adams, E. W.: Histological and Histochemical Observations on Bovine Teat Epithelium. M.S. thesis, Cornell University, 1957.

[2] Barcroft, J., McAnally, R. A., and Phillipson, A. T.: Absorption of Volatile Fatty Acids from the Alimentary Tract of the Sheep and Other Animals. J. Exptl. Biol., *20*, (1944): 120–129.

[3] Barcroft, J., McAnally, R. A., and Phillipson, A. T.: The Absorption of Sodium Ortho-Iodo-Hippurate from the Rumen of Lambs. J. Exptl. Biol., *20*, (1944): 132–133.

[4] Basu, S. L., Sen, R., and Ghosh, A.: Some Histological and Histochemical Observations of the Caprine Forestomach. Indian J. Physiol., *11*, (1957): 21–26.

[5] Cain, A. J.: The Use of Nile Blue in the Examination of Lipoids. Quart. J. Micros. Sci., *88*, (1947): 383–392.

[6] Carleton, H. M., and Drury, R. A. B.: Histological Technique. 3rd ed. Oxford Press, London, 1957.

[7] Conrad, H. R., Smith, H. R., Vandersall, J. H., Pounden, W. D., and Hibbs, J. W.: Estimating Gastrosplenic Blood Flow and Volatile Fatty Acid Absorption from the Forestomachs of Calves. J. Dai. Sci., *41*, (1958): 1094–1099.

[8] Dobson, M. J., Brown, W. C. B., and Phillipson, A. T.: A Histological Study of the Organization of the Rumen Epithelium of Sheep. Quart. J. Exptl. Physiol., *41*, (1956): 247–253.

[9] Ellenberger, W.: Handbuch der vergleichenden microskopischen Anatomie der Haustiere. Vol. 3. Paul Parey, Berlin (1911): 144, 157, 162.

[10] Flatt, W. P., Warner, R. G., and Loosli, J. K.: The Influence of Purified Materials on the Development of the Ruminant Stomach. J. Dai. Sci., *41*, (1958): 1593–1600.

[11] Gobetto, A.: Morfogenesi ed istogenesi della mucosa del rumine. Atti Soc. ital. sci. vet., *11*, (1957): 782–787.

[12] Gobetto, A.: Morfogenesi ed istogenesi della mucosa del reticolo e dell' omaso. Ann. d. Fac. med. vet., *11*, (1958): 1–22.

[13] Gömöri, G.: Microscopic Histochemistry. Univ. Chicago Press, Chicago (1952): 96–97.

[14] Hauser, H.: Über interessante Erscheinungen am Epithel der Wiederkäuervormägen. Ztschr. mikr.-anat. Forsch., *17*, (1929): 533–612.

[15] Montagna, W.: The Structure and Function of Skin. Academic Press, New York, N.Y., 1956.

[16] O'Flaherty, F., Roddy, W. T., and Lollar, R. M.: The Chemistry and Technology of Leather. Vol. I. Reinhold Publishing Corp., New York, N.Y., 1956.

[17] Pennington, R. J.: The Metabolism of Short-Chain Fatty Acids in the Sheep. I. Fatty Acid Utilization and Ketone Body Production by Rumen Epithelium and Other Tissues. Biochem. J., *51*, (1952): 251–258.

[18] Pennington, R. J., and Pfander, W. H.: The Metabolism of Short-Chain Fatty Acids in the Sheep. 5. Some Interrelationships in the Metabolism of Fatty Acids and Glucose by Sheep-Rumen Epithelial Tissue. Biochem. J., *65*, (1957): 109–111.

[19] Weber, A. F., Phillips, M. G., and Bell, J. T., Jr.: An Improved Method for the Schultz Cholesterol Test. J. Histochem. & Cytochem., *4*, (1956): 308–309.

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Trial with a New Organic Phosphate as an Anthelmintic in Sheep

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THE RECENT widespread interest in organic phosphorus compounds as insecticides and miticides, particularly with respect to their systemic activity in mammals, has led several investigators to explore their possible value as anthelmintics.

Levine *et al.*[7-9] reported that several compounds of this class showed nematocidal activity against horse strongyle larvae *in vitro*, and one, 0,0-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (Dipterex*), was reported to have removed the majority of gastrointestinal nematodes from a sheep when used at 400 mg./kg. of body weight. This dose rate, however, produced a typical phosphate toxicosis.

Herlich and Johnson,[4] in a critical test of the anthelmintic efficiency of 0, 0-dimethyl 0-2,4,5-trichlorophenyl phosphorothioate (Trolene†) in cattle, reported that a dose rate of 100 mg./kg. of body weight yielded rather variable results against the species of nematodes encountered. The drug was quite effective against *Haemonchus placei* (98%) and less so against *Cooperia punctata* (59%), *Ostertagia ostertagi* (46%), *Oesophagostomum radiatum* (30%), and Trichuris spp. (9%). *Trichostrongylus axei*, *Trichostrongylus colubriformis*, and *Nematodirus helvetianus* were essentially unaffected by the treatment.

Schad *et al.*[11] treated 5 aged ewes with this drug at the rate of 100 mg./kg. of body weight and found no evidence of anthelmintic action against Haemonchus, Ostertagia (Pseudostertagia), Trichostrongylus, Nematodirus, and Chabertia. The compound also appeared to have no effect on tapeworms of the genus Thysanosoma.

Recently, Herlich and Porter[6] reported their results with another organic phosphate compound, 0,0, diethyl 0-(3-chloro-4-methyl-7-coumarinyl) phosphorothioate (Bayer 21/199**). In cattle and sheep, when administered at the rate of 25 mg./kg. of body weight, this drug was highly efficient against species of the genera Haemonchus, Ostertagia, Trichostrongylus, Cooperia, Trichuris, and Capillaria, and *Strongyloides papillosus*. It was erratic or ineffective against Nematodirus and *Bunostomum phlebotomum*. Mild and transitory signs of intoxication were seen in some of the treated animals, leading the authors to suggest that the 25 mg./kg. dose level should probably not be exceeded.

Baker *et al.*[1] used an experimental organic phosphorus compound against gastrointestinal nematodes in cattle at the rate of 120 to 130 mg./kg. of body weight. On the basis of reduction in worm numbers in 10 treated animals as compared with a like number of untreated animals, they reported an over-all efficiency of 71 per cent with a 70 per cent efficiency in the abomasum and 88 per cent in the small intestine. The drug was most effective against Trichostrongylus (96–100%), slightly less effective against Cooperia (87%), and least effective against Ostertagia (66%), which made up 82 per cent of the total worm population in the untreated animals.

In view of the results reported with this compound and the apparent failure of related compounds when used in sheep,[11] it seemed desirable to investigate the effect of this material on the gastrointestinal nematodes of sheep.

MATERIALS AND METHODS

Range lambs with natural infections were placed in a dry lot and fed alfalfa hay. On the basis of five fecal worm egg counts made over a period of five weeks, they were assigned to groups of 10 lambs each, in which the average worm egg count was essentially the same, as was the distribution of counts within the groups.

The experimental organic phosphorus compound, 0-methyl 0-(4-tert. butyl-2-chlorophenyl) ethylphosphophoramidothiate (Dowco 105*), was used in the form of a 25 per cent wettable powder and was administered by stomach tube. Animals were treated with the compound as follows: group 1, 200 mg./kg.; group 2, 75 mg./kg.; and group 3, no treatment.

Three fecal egg counts were made following treatment and, on the sixth day, the lambs were slaughtered. Abomasums and small intestines were collected and worm counts made by methods previously described.[2]

RESULTS

The effect of treatment on the worm egg counts and the worm population is shown (tables 1, 2). The efficiency of treatment was calculated on the basis of percentage

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*Dipterex is available from Chemagro Corp., New York, N.Y.

† Trolene (Dow-ET-57) is produced by the Dow Chemical Co., Midland, Mich.

** Bayer 21/199 is available from Chemagro Corp., New York, N.Y.

* Dowco 105 is produced by Dow Chemical Corp., Midland, Mich.

TABLE 1—The Effect of Treatment on Worm Egg Counts

Group	Pretreatment average	Post-treatment average	Reduction (%)
1 (200 mg./kg.)	479	52	89
2 (75 mg./kg.)	523	169	68
3 (no treatment)	503	412	18

reduction in worm numbers in the treated lambs as compared with the controls. Analysis of the difference observed between the mean worm counts in animals of the treated and untreated groups, using Student's "t" test, indicated that the differences between the treated sheep and between sheep in the 200 mg./kg. group and the untreated group are highly significant ($P = >0.001$). The differences between animals in the 75 mg./kg. group and the untreated group were found to be significant at the 5 per cent confidence level.

The variation in anthelmintic efficiency of the compound against the species of nematodes present in the lambs is shown (table 4). In table 4, the two species of Ostertagia were combined and Haemonchus was omitted, since this genus represented less than 1 per cent of the population in the untreated group.

DISCUSSION

In contrast with the experience of Baker *et al.*,[1] in which they reported a 50 per cent incidence of intoxication, which was mild and transitory except for 1 animal, none of the animals in this trial showed any evidence of toxicosis due to the treatment. This finding supports the results of Radeleff and Woodward,[10] who reported that a closely related compound was much less toxic to sheep than to cattle.

In general, the results reported here are comparable with those reported by Baker *et al.*[1] This drug appears to be significantly more effective against the species of nematodes inhabiting the small intestine than it is against those found in the abomasum. On the whole, at the rate of 200 mg./kg. of body weight, it is more efficient against the species of Trichostrongylus than it is against the species of Ostertagia. A notable exception is *T. axei*, which was only poorly controlled by the higher dose and apparently was not affected by the 75 mg./kg. dose level. No explanation of this result can be offered on the basis of the experiment

TABLE 2—Average Total Worm Count and Percentage of Efficiency of Treatment in the Abomasums and Small Intestines of Lambs

Group	Abomasum	Efficiency (%)	Small intestine	Efficiency (%)	Total	Efficiency (%)
1 (200 mg./kg.)	116	91	18	98	134	93
2 (75 mg./kg.)	643	48	246	67	889	55
3 (no treatment)	1,241	...	755	....	1,996	....

The relative abundance, in percentage of the total population, of the various species of nematodes encountered in the abomasums and small intestines of the lambs is indicated (table 3). Specific identifications were made on male trichostrongylids; females were then assigned to a particular species on the basis of relative abundance of the males. This necessarily requires the assumption that sex ratios of the species within a genus are the same.

TABLE 3—Percentage Distribution of Species of Nematodes in the Abomasums and Small Intestines of Lambs

Species	200 mg./kg.	75 mg./kg.	No treatment
Abomasum			
Ostertagia circumcincta	56	72	64
O. trifurcata	4	9	23
Trichostrongylus axei	40	18	6
T. vitrinus	0	0	7
Haemonchus sp.	0	1	0
Small intestine			
T. vitrinus	12	33	45
T. colubriformis	12	27	7
Strongyloides papillosus	58	29	30
Nematodirus filicollis	9	7	6
N. spathiger	9	4	12

TABLE 4—Percentage Anthelmintic Efficiency by Species in the Abomasums and Small Intestines of Lambs

Species	200 mg./kg.	75 mg./kg.
Abomasum		
Ostertagia spp.	94	52
Trichostrongylus axei	38	0
T. vitrinus	100	100
Small intestine		
T. vitrinus	99	76
T. colubriformis	96	0
Strongyloides papillosus	95	69
Nematodirus filicollis	96	62
N. spathiger	98	89

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and it should be subjected to further investigation.

It is of interest that Herlich and Porter [6] reported Bayer 21/199 to be highly efficient in removing *Strongyloides papillosus* and that their results parallel those obtained in this trial. As these authors pointed out, this was, " . . . the first experimental evidence of highly effective anthelmintic activity resulting from a single oral administration of a drug against any species of Strongyloides."

In the trials which have been reported on the anthelmintic activity of the organic phosphorus compounds, species of the genus Nematodirus have not been affected at any dose level employed.[4-6] This is in contrast with the experience reported here, in which the degree of control of Nematodirus was excellent at the 200 mg./kg. level and reasonably satisfactory at 75 mg./kg. (table 4).

Although the lower dose level produced somewhat erratic results, the over-all efficiency was comparable with that reported for commercial phenothiazine preparations when used under similar conditions.[3,4] In the experience of the authors, the degree of efficiency attained with the higher level of administration is far superior to that obtained with commercial phenothiazine preparations and is comparable with a 625 to 1,250 mg./kg. dosage of fine-particle purified phenothiazine.

These results, and those reported by other investigators, suggest that this class of compounds offers considerable promise as anthelmintics for use in ruminants and possibly other species of animals. Their spectrum of activity is broader than other drugs, such as phenothiazine, and they are generally efficient at much lower levels of administration. However, the problem of toxicity must be investigated further before definite recommendations for use can be made.

In the experience of the authors, the incidence of intoxication resulting from cholinesterase inhibition can not be predicted with accuracy. Severely parasitized and debilitated cattle, for example, may show no evidence of the toxicity of Bayer 21/199 when it is given at the rate of 20 mg./kg., whereas animals from the same herd in much better physical condition may show an incidence of intoxication of 30 per cent or more when they are given the same dose. Although the compound used in the preceding experiment appears to be somewhat less toxic for cattle than Bayer 21/199, in the authors' experience (unpublished) much more information is required in order to understand this problem.

SUMMARY

An organic phosphorus compouud, Dowco 105, was found to have marked anthelmintic activity against most species of nematodes in the abomasum and small intestine of lambs. A dosage rate of 200 mg./kg. of body weight gave an over-all anthelmintic efficiency of 93 per cent. When the dose was reduced to 75 mg./kg., anthelmintic efficiency dropped to 55 per cent.

It is considered that compounds of this class show promise as anthelmintics for use in ruminants and possibly other species of animals.

References

[1] Baker, N. F., Allen, P. H., and Douglas, J. R.: Trial with a New Organic Phosphate as an Anthelmintic in Cattle. Am. J. Vet. Res., *20*, (March, 1959): 278–280.

[2] Douglas, J. R., Baker, N. F., and Longhurst, W. M.: The Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine. Am. J. Vet. Res., *17*, (April, 1956): 318–323.

[3] Douglas, J. R., Baker, N. F., and Longhurst, W. M.: Further Studies on the Relationship Between Particle Size and Anthelmintic Efficiency of Phenothiazine. Am. J. Vet. Res., *20*, (Jan., 1959): 201–205.

[4] Fritts, D. H., Hawkins, W. W., and Marquardt, W. C.: A Comparison of Three Anthelmintic Preparations Against Nematodes of Sheep. J.A.V.M.A., *133*, (July 15, 1958): 113–115.

[5] Herlich, H., and Johnson, J. M.: Critical Tests on the Efficiency of Dow-ET-57 as an Anthelmintic in Cattle. J. Parasitol., *43*, (suppl.) (1957): 19.

[6] Herlich, H., and Porter, D. A.: An Anthelmintic for Cattle and Sheep: Critical Tests of Efficacy of Bayer 21/199. Vet. Med., *53*, (1958): 343–348.

[7] Levine, N. D., Ivens, V., Kleckner, M. D., and Sonder, J. K.: Nematocidal Screening Tests of Organic Phosphorus, Nitrofuran, Cadmium and Other Compounds Against Horse Strongyle Larvae *in Vitro*. Am. J. Vet. Res., *17*, (Jan., 1956): 117–120.

[8] Levine, N. D., Kantor, S., and Taylor, G. D.: Nematocidal Activity of Some Organic Phosphorus Compounds Against Horse Strongyle Larvae *in Vitro*. Am. J. Vet. Res., *19*, (April, 1958): 299–303.

[9] Levine, N. D., Kantor, S., and Taylor, G. D.: Trials of Organic Phosphorus Nematocides in Sheep and Mice. Illinois Vet., *1*, (1958): 6–9.

[10] Radeleff, R. D., and Woodward, G. T.: Toxicological Studies of Dow-ET-57 in Cattle and Sheep. J. Econ. Entomol., *50*, (1957): 249–251.

[11] Schad, G. A., Allen, R. W., and Samson, K. S.: The Effect of Dow-ET-57 on Some Sheep Parasites. Vet. Med., *53*, (1958): 533.

Increases in the Frothing of Samples of Ruminal Digesta Following the Addition of Certain Nonvolatile Organic Acids of Legumes

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AN EXTENSIVE TRAPPING of gas in froth in the rumen, with its consequent inhibition of the elimination of sufficient gas by eructation to maintain normal pressures in the rumen, is receiving greater consideration as the probable reason for the development of legume bloat.

The production of gas from ruminal digesta and the process of frothing are results of normal digestion. The normal ruminant can eructate volumes of free gas far in excess of what is normally produced on any known feeding regimen. If, however, the gas of an active gas-producing ruminal digesta is retained in rapidly increasing amounts in froth which becomes increasingly more stable during the same period, the eventual result is the failure of the pressure-release mechanisms of the rumen. Ruminants which normally eructate free gas to release pressure will not eructate froth. The gas of the froth continues to be retained, therefore, until factors which determine the breaking rate of the froth exceed those which determine its rate of production. Death may occur during the period of froth production if withstandable pressures within the rumen are exceeded. It is necessary to define the reasons for the trapping of gas if a final definition of the cause of legume bloat is to be written.

One of the many factors which contribute to an increase of the volume of a froth is the rate of formation of bubbles. Gas must be produced to make the bubbles of the froth. If gas can be produced with sufficient rapidity in ruminal contents, it could increase the maximum volume of froth produced even if froth-breaking rates were normal at the onset of gas production and even if the volumes of gas produced at the time of maximum frothing were not greater than normal. Any factor which may be present at the onset of rapid gas production or which may develop during such production and which lengthens froth-breaking times also contributes to a larger volume of froth. For example, the feeding of fresh legumes results in more viscous prefeeding ruminal contents than the feeding of alfalfa

TABLE 1—Comparison of the Means of the 60- and 120-Minute and Maximum Controls and the Means of the 120-Minute and Maximum Values of Froth Gas, Froth, Gas, Free Gas, and Liquid of Ten 50-ml. Samples of Filtered Ruminal Fluid with Concentrations of Organic Acids Added to a Final Concentration of M/100

	Time (min.)	Froth gas	Froth	Gas	Free gas	Liquid
Control	60	3.5	12.6	7.0	3.5	40.9
Malonic	59(m)	11.3**	17.2**	12.9**	1.6**	44.1**
Malic	65(m)	12.1**	17.6**	14.1**	2.0	44.5**
Citric	54(m)	14.0**	19.8**	15.7**	1.7*	44.2**
Succinic	65(m)	9.6**	15.8*	12.9**	3.3	43.8**
Control	120	5.9	12.6	11.5	5.6	43.3
Malonic	120	6.0	11.2	21.3**	15.3**	44.8*
Malic	120	6.4	12.1	22.9**	16.5**	44.3
Citric	120	5.8	10.7*	26.3**	20.5**	45.1**
Succinic	120	6.7	11.8	19.3**	12.6**	44.9**
Control	155(m)	6.3	12.9	12.7	6.4	43.4
Malonic	59(m)	11.3**	17.2**	12.9	1.6**	44.1
Malic	65(m)	12.1**	17.6**	14.1	2.0**	44.5
Citric	54(m)	14.0**	19.8**	15.7	1.7**	44.2
Succinic	65(m)	9.6**	15.8*	12.9	3.3**	43.8

Values are given in milliliters. (m) = time of maximum increase in volume of sample or maximum FG.
* significant at 5 per cent. ** significant at 1 per cent.

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hay. Surface tensions of ruminal contents are increased also by the feeding of fresh legumes.[1,2]

Rosen *et al.*[3] demonstrated that the addition of certain organic acids, *i.e.*, citric, succinic, malic, and malonic acids, to samples of active ruminal digesta in Warburg flasks resulted in an increased rate of production of CO_2. Eluted portions of extracts of legumes, chromatographically similar to these acids, produced CO_2 in a similar manner. Many other metabolic compounds and similarly prepared extracts of nonlegumes in similar concentrations failed to produce CO_2 with such rapidity. Increased frothing of samples of ruminal digesta was observed during the production of CO_2 but was not measured at the time.

MATERIALS AND METHODS

On ten different days, samples of strained ruminal fluid were taken from the same region of the rumen of the same fistulated cow 30 minutes after the morning feeding of alfalfa hay. A 50.0-ml. portion of each sample was retained as a control. To a similar 50.0-ml. portion was added a quantity of one of the previously mentioned acids, calculated to equal a final concentration of M/100 and capable of releasing 11.2 ml. of CO_2. The quantities added were 26.0 mg. of malonic, 33.5 mg. of malic, 32.0 mg. of citric and 29.5 mg. of succinic acids. Each portion of each sample was incorporated immediately into an apparatus (fig. 1) for measuring gas produced by the sample. The following measurements (in ml.) were made at intervals of 5, 15, 30, 60, 90, and 120 minutes; some also at 150 and 240 minutes: volume of froth gas (FG), or the increase in volume of the sample; volume of froth (F), or the amount of froth floating on the liquid of the sample; and volume of gas (G) or the amount of displaced confining fluid. The following were ascertained for each portion of each sample also: maximum volume of froth gas, or the maximum increase in volume of the sample before the froth began to break; maximum volume of froth, or the amount of froth at the time of maximum increase in volume of the sample; volume of gas at the time of maximum increase in the volume of the sample; free gas or G − FG; free liquid (L), or 50 + FG − F; and analyses of appropriate mean differences.

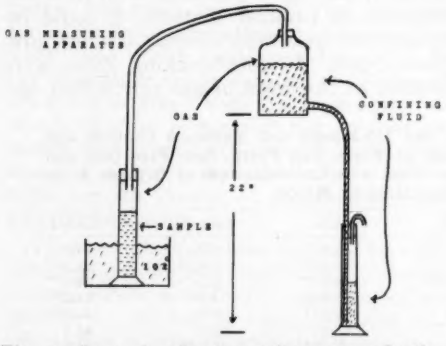

Fig. 1—Apparatus for measuring gas production by a sample of ruminal contents.

As gas is formed in the sample, the pressure in the closed portion of the apparatus (designated "gas") increases, allowing some of the confining solution in the upper container to gravitate into the graduated cylinder (right) below. The amount of confining fluid entering the graduated cylinder represents the amount of gas produced under these conditions. Measurements of gas formed by samples to which the organic acids have been added are always compared with those of untreated portions of the same samples as controls.

The cylinder containing the sample is also graduated, allowing measurements of the increases in the volumes of the samples due to froth gas produced by the formation of bubbles within the samples.

The amount of froth which comes to the surface of the sample is measured by subtracting the volume of separated liquid of the sample from the total volume of the sample.

The plotting of frequent readings of the total expansion of the sample will yield measurements of maximum froth gas.

RESULTS

Examination of the means (table 1) indicates that the added acids produced:

1) Increased maximum volumes of froth; 17.2, 17.6, 19.8, 15.8 vs. 12.9 ml.;

2) Earlier maximum volumes of froth; 59, 65, 54, 65 vs. 155 minutes;

3) Increased maximum volumes of froth with no significant increases in the volumes of gas produced at the times of maximum frothing; 12.9, 14.1, 15.7, 12.9 vs. 12.7 ml.;

4) Maximum frothing before evidence of completion of the final increases in volumes of gas which might be expected to result from a simple release of CO_2 (11.2 ml.) from the acids added; 21.3, 22.9, 26.3, 19.3 vs. 11.5 ml.

SUMMARY

Quantities of malonic, malic, citric, and succinic acids equivalent to a final concentration of M/100 added to 50-ml. portions of strained fresh ruminal fluid increased the rates of formation of froth and thus the volumes of froth during the periods of more rapid formation of the froth.

References

[1] Nichols, R. E., Dillon, R. D., Penn, K., Bryant, J., and Schreiber, J.: The Apparent Surface Tension and Relative Viscosity of Paunch Fluid of Cattle Fed Hay, Fresh Grass or Fresh Alfalfa. Vet. Med., *51*, (1956): 389–391.

[2] Nichols, R. E., Penn. K., and Schreiber, J.: Frothing, Surface Tensions and Viscosities of Paunch Contents Associated with Changes from Alfalfa Hay to Fresh Legumes. Vet. Med., *52*, (1957): 37–40.

[3] Rosen, W. G., Fassell, H., and Nichols, R. E.: The Effects of Carboxylic Acids on Gas Production by Rumen Microorganisms and Its Possible Relation to Legume Bloat. J. Anim. Sci., *15*, (1956): 1304.

XUM

Radiographic Diagnosis of Mesenteric Abscesses in the Horse

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AMONG the different growths which may occur in the abdominal cavity of the horse, abscesses in the anterior mesenteric root are especially significant.

ETIOLOGY

The cause of such abscesses has not been fully investigated, since isolation of the causative agents can be performed only at necropsy. Also, in protracted chronic conditions, such abscesses might be bacteriologically sterile in consequence of autosterilization.

Findings, up to the present, indicate that such abscesses most often represent infection with various strains of streptococci, among which *Streptococcus equi*, the causative agent of strangles (Adenitis equorum), occupies a special position with regard to its incidence and importance. It has been often observed, in the course of typical strangles, that metastases and a spread of infection to various parts of the body may develop, with a most frequent localization in the mesenteric lymph nodes, *i.e.*, the so-called "abdominal strangles." Abscesses in abdominal strangles are generally chronic and may attain the size of a human head, with a wall several millimeters to a centimeter in thickness.

Abscesses in the mesenteric root may occasionally be produced by other types of bacteria, such as *Shigella equirulis* (B. pyosepticum viscosum equi), which is known to cause a septicemia in the horse and which has been isolated also from verminous aneurysms in mature horses. In such cases, the presence of abscesses was often established on the external side of a parasitic thrombus in the anterior mesenteric root.[4] *Salmonella typhimurium* has also been found in many species of animals and has been isolated from purulent subdermal abscesses in foals and from abscesses in the mesenteric root in horses.

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CLINICAL SIGNS

Such abscesses usually develop in the later course of the disease (weeks, months, and even years after a case of typical strangles) and, in some cases, without any earlier sign of disease in the submandibular lymph nodes. Rectal examination of the mesenteric root reveals an elastic swelling of varying size, more or less mobile, and which might even fluctuate. The newer the process, the greater will be its sensitivity on palpation. A careful anamneses usually reveals various digestive upsets, temporary diarrhea alternating with hard small excrement, and signs of constipation.

Mesenteric abscesses may cause a secondary stenosis of the intestines, due to external pressure, which can activate brief attacks of colic, particularly if the animal is not properly fed. However, a pregnant uterus, an enlarged bladder, various tumors, and hematomas may also give rise to secondary stenosis of the intestines. As passage through the bowels grows more difficult, the animal's condition gradually declines, its body temperature is permanently raised, and finally purulent peritonitis may develop as a sequela to perforation of the abscess. In rare cases, the contents of the abscess may be discharged into the lumen of the intestine and the animal may recover spontaneously.

According to Szöllös,[8] primary involvement of the mesenteric lymph nodes may reveal itself by signs of acute gastroenteritis of varying degree and outcome. Aside from colic, there is tenderness in the region of the xiphoid cartilage and restlessness when defecating or urinating. Mesenteric abscesses usually form adhesions to the proximal loops of the intestine, other organs, or the abdominal wall with, in consequence, disturbances of digestion. On the basis of his observations in eight clinical cases, Sutlić [7] pointed out that involution of atypical forms of strangles, with a localization in the mesenteric lymph nodes, may take a different course from the formerly accepted one. Quite often, swellings of generous size (if they do not fluctuate on the surface)

Fig. 1—Mesenteric abscess in the shape of a hemisphere.

Fig. 2—Mesenteric abscess in the shape of an ellipse.

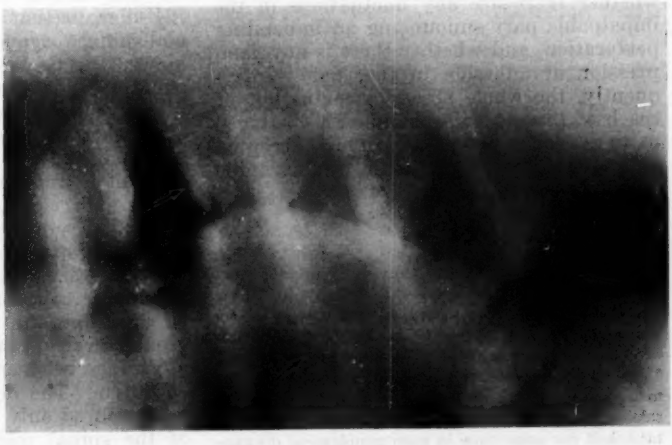

Fig. 3—Mesenteric abscess in the shape of a cone.

may disappear in a relatively short time without any treatment, thus implying that the lymph nodes do not become purulent in every case but that the swelling appears in consequence of collateral edema and infiltration with leukocytes. The real cause of the disease is often revealed as a chance finding at necropsy.

DIAGNOSIS

Salmonellosis may be considered in differential diagnosis, but it can be eliminated by serological examination. Infectious anemia is more difficult to exclude because in this disease, also, the body temperature is raised, the mucosae are pale, the animal becomes emaciated, and undefined clinical signs are manifested.

Kovač [3] emphasized the value of blood counts in horses suffering from strangles and concluded that the erythrocyte count is reduced during the ripening of the abscess, while the number of erythrocytes increases parallel with the general improvement of the animal's condition after the abscess has opened and the infiltrate has been absorbed.

Rectal examination is the means not only for establishing the presence of mesenteric abscesses, but also of ascertaining their topographical position, size, form, consistency, sensitivity, fluctuation, the compression of organs, and adhesion to their environment. However, quite often only the caudal region of the growth can be reached with the examining hand. In such cases, it is impossible to discover how far the abscess extends in the cranial direction, whether there are any fluctuations in the impalpable part announcing an impending perforation, and whether there is any compression or adhesion in that part. Infrequently, the whole abscess remains out of reach of the exploring hand. This induced us to endeavor to complement the accepted diagnostic procedure with radiographic examinations.

MATERIALS AND METHODS

Roentgenological dissociation of abdominal organs in the horse is impossible because of their practically equal absorptivity, as well as because of the volume of the abdomen; hence the application of gas as a contrast in the abdominal cavity seemed a reasonable procedure. Artificial pneumoperitoneum as a method of choice for obtaining better selectivity in the abdominal organs has been established for some time in roentgenological diagnosis in small domestic animals [2, 6] and cattle. [1, 9] In the case of horses, the method has been adopted only in its experimental stage by Lukšić. [5]

The animal was prepared for artificial pneumoperitoneum by fasting for two to three days. At the same time, to evacuate the digestive tract as thoroughly as possible, the digestive tract was systematically emptied by mild laxatives (magnesium and sodium sulfate) and, when the bowel contents became mushy, evacuation was accelerated by repeated small doses of carbamyl choline chloride. Before performing rectal examination, epidural anesthesia was applied. Finally, it was found useful to administer a barbiturate (sodium phenobarbital) intravenously or intramuscularly in doses of 2 to 5 Gm., thus calming the animal completely.

Following the method of Lukšić, [5] air was insufflated into the peritoneal cavity through a needle by means of an air suction pump combined with a filter. The abdomen was punctured on the left side, caudal to the last rib and 6 to 8 cm. ventral from the transverse processes of the lumbar vertebrae. The left flank was used in order to circumvent possible perforation of the cecum, but only after determining, by palpation, the exact position of the spleen. When the needle pierced the skin, it was thrust rapidly into the peritoneal cavity.

Small horses were insufflated with 10, medium-sized horses with 15, and large animals with 20 liters of air. During insufflation, the respiration and pulse rates were checked and the animal was closely observed. Horses usually tolerated the insufflation and pneumoperitoneum with equal ease.

The pulse rate underwent little change until toward the end of the application, when the pulse became somewhat harder. When the diagnostic procedure ended, most of the air was released from the abdominal cavity. Although untoward sequelae were seldom observed, wide-spectrum antibiotics were administered to prevent possible complications.

DISCUSSION

Under present technical conditions, the abdominal organs in the horse can be viewed in profile only with the animal standing. The radical evacuation of the digestive tract and copious insufflation of air makes it possible to observe the unchanged anterior mesenteric root in the form of a transparent band, about 10 cm. wide, with clear, sharp outlines running vertically, ventral to the eighteenth thoracic and the first and second lumbar vertebrae. Abscesses are contrasted against the light pneumatic zone in their entire mass, both on screening and on the plate. They vary greatly in size; craniocaudal diameters usually range between 10 and 20 cm., but may be 30 cm. The dorsoventral diameter can be measured only from the ventral outline of the spinal column because the rest is covered and ranges, according to our lim-

ited experience (8 animals), from 6 to 20 cm.

Although the shape of mesenteric abscesses varies greatly, they are projected in the pneumatic zone most often as segments of a sphere or hemisphere, the base of which is located along and in part across the vertebrae. The greater the vertical diameter of the abscess the more it approaches in shape a semi-ellipse or a blunt cone, the apex of which is directed ventrally. Less frequently, mesenteric abscesses are projected in the shape of an absolutely regular ellipse. The sharp, smooth outlines are most often regularly convex. The ventral outline generally presents a more or less definite waviness with conical or quite irregular protuberances. The dorsal outline usually can not be distinguished.

Mesenteric abscesses may extend from the fourteenth rib to the fourth lumbar vertebra. In accordance with the degree of evacuation of the digestive tract and the quantity of insufflated air, the ventral portions of mesenteric abscesses either hang in the free pneumatic zone or lie in part against the liver, stomach, right dorsal loop of the large colon and, in exceptional instances, some loops of the empty intestine.

The density of the shadow of such abscesses corresponds to the soft parts but lacks homogeneity. Abscesses are most often shadowed where they are covered by the spinal column, by the dorsal part of the right lobe of the liver, and by the kidneys. If the stomach and duodenum contain gas, a marked reduction of the shadow in the cranioventral part of the abscess may result. A similar reduction will ensue in the caudoventral part from a gas-filled cecum and from meteorism of the right dorsal portion of the large colon if they are projected across the abscess. In rare cases, particularly in insufficient evacuation of the digestive tract, a part of the filled large colon may cover the ventral segment of the abscess. Such a nonhomogenous shadow makes it difficult to estimate whether mesenteric abscesses contain gas.

For reasons of economy in practical routine diagnostics, fluoroscopy has greater value than the far more expensive radiography.

Summary

The diagnosis of mesenteric abscesses in the horse can be based upon rectal examination; however, this may be unsatisfactory, since it rarely allows for palpation of the entire growth. Generally, only the caudal portion of the abscess is accessible and sometimes the whole abscess lies out of reach of the exploring hand.

Radiological examination, accompanied by a copious artificial pneumoperitoneum, makes it possible to view the size, shape, location, and relation toward proximal organs of these abscesses, regardless of their topographical location.

The animal is prepared for this examination by being deprived of its feed for two to three days and by application of mild laxatives. After sedation by means of a barbiturate, air is insufflated through the left flank—10 liters in small horses, 15 liters in medium-sized horses, and 20 liters in large animals. After the examination, most of the air is released. The animals exhibit no unfavorable aftereffects.

References

[1] Dusinović, M.: Artificijalni pneumoperitoneum goveda kao pomoćno sredstvo u rentgenskoj dijagnostici. Veterinaria (Sarajevo), *3*, (1954): 604–615.

[2] Gošler, K.: Rentgensko istrazivanje gravidnosti u kunića. Vet. Arhiv., *22*, (1952): 139–150.

[3] Kovac, F.: Krvna slika i toksicne promjene neutrofilnih leukocita kod zdrebećaka i štenećaka. Vet. Arhiv., *3*, (1933): 1–80.

[4] Langhoff, L.: Infektion mit dem Bakterium pyosepticum viscosum equi unter besonderer Berücksichtigung der makroskopischen, pathologisch-anatomischen Erscheinungen. Berl. u. Münch tierärztl. Wchnschr., *39*, (1923): 358–359.

[5] Lukšić, A.: Rentgensko prikazivanje trbušnih i nekih okolnih organa kod konja s pomoću pneumoperitoneuma. Vet. Arhiv., *22*, (1952): 157–170.

[6] Sartoris, P.: Il pneumoperitoneo nella radiodiagnostica della gravidanza dei carnivori domestici. Ann. radiol. fisica medica, *9*, (1933): 171–188.

[7] Sutlić, A.: Prinos poznavanju abdominalnog oblika zdrebećaka. Vet. Arhiv., *2*, (1932): 476–504.

[8] Szöllös: Spezielle Pathologie und Therapie der Haustiere. F. Hutyra, J. Marek, and R. Manninger, ed. (1938): 345.

[9] Westhues, N.: Die röntgenologische Fremdkörperdiagnose beim Rinde und Bemerkungen zur röntgendiagnostischen Überwindung dicker Objekte. Berl. u. Münch. tierärztl. Wchnschr., *46*, (1930): 565–568.

XUM

Field Tests on the Efficacy of Piperazine-Carbon Disulfide Complex in the Treatment of Foals for Gastrointestinal Parasites

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CRITICAL TESTS and field trials indicate that the piperazine-carbon disulfide complex (Parvex*) is very effective as an ascaricide in swine.[3,6] This compound was shown by Poynter[4,5] to be an effective anthelmintic against *Parascaris equorum* and several small strongleyes when given to horses at 100 mg. per kilogram of body weight. Field trials[1] and critical tests with grade doses[2] show that Parvex Suspension* has activity in removing *P. equorum*, *Oxyuris equi*, strongyles, and stomach bots from horses.

This paper is a report on the efficacy of this suspension in removing gastrointestinal parasites from foals, based on results obtained in field trials, and the hematology of foals treated with the suspension.

MATERIALS AND METHODS

Forty-seven nursing foals, 26 to 147 days old and weighing 225 to 485 lb., were allotted to three groups of 14 foals and one group of 5 foals, so that the average age and age range of the foals were approximately the same in each group. The animals in each group of 14 foals were given a different dosage level of the suspension; group 1 at the rate of 150, group 2 at 100, and group 3 at 75 mg./kg. of body weight. The 5 foals in group 4 served as controls; however, 4 of these foals were later given 50 mg. of the suspension per kilogram of body weight, and this group of 4 was designated as group 4A. The remaining foal (F-6) of the original control group was later given 12 ml. of carbon disulfide, followed two weeks later by 100 mg. of the suspension per kilogram of body weight. A group of 7 foals, designated as group 7 and made up of 2 foals from group 1, 3 from group 2, and 2 from group 3, were each given 12 ml. of carbon disulfide two weeks after treatment with the suspension.

All doses were measured in a glass syringe and given by oral intubation, followed by a 250-ml. oral water flush.

Before treatment, the foals and dams were confined overnight, with water and hay available. On the day a group was treated, approximately two hours were devoted to collecting fecal samples and dosing, and two hours to observing the animals after treatment. After dosing, the foals and mares were fed, watered, and turned out to pasture for approximately five hours. At that time, each foal with its dam was placed in a box stall with feed and water for 45 hours. An attempt was made to collect all feces from each foal during this 45-hour period.

All feces collected after treatment were screened through a series of $\frac{1}{2}$-, $\frac{1}{4}$-, $\frac{1}{16}$-inch and No. 40-gauge wire meshes. All ascarids, pinworms, grossly visible strongyles, and bot larvae were collected from the screens. Species and stage of bot larvae were identified.

Two pretreatment and two post-treatment egg counts were made on each foal, using fecal samples collected from the rectum. One pretreatment sample was collected approximately one week before treatment and another immediately before dosing an animal. The post-treatment samples were collected seven to eight days after treatment and approximately one month after treatment. The number of helminth eggs per gram (e.p.g.) of feces was determined by diluting 4 Gm. of feces in 56.0 ml. of water in a Stoll flask. One milliliter of this mixture was diluted in 1.0 ml. of concentrated sugar solution. One milliliter of the second dilution was divided into 0.5-ml. portions, placed into two counting chambers, and covered with a coverglass. Each egg counted represented 30 e.p.g. of feces. This method consistently gave reproducible counts for Parascaris and strongyle eggs.

Fecal cultures were made using fecal samples collected from 10 foals to determine whether large or small strongyle eggs were being passed in the feces.

Duplicate hemograms, leukocyte counts, erythrocyte counts, hemoglobin determinations, sedimentation rates, and hematocrits were determined on all foals before treatment. Two days after treatment, the above determinations were made in duplicate on at least half of each treated group. Approximately 30 days after treatment, duplicate determinations of the above tests were again completed on all foals that had not been sold.

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* Parvex (a complex of betaine 1-piperazine carbodithioic acid) and Parvex Suspension (a suspension containing 250 mg./cc. of betain 1-piperazine carbodithioic acid) are produced by the Upjohn Company, Kalamazoo, Mich.

TABLE 1—Parascaris and Strongyle Egg Count Data on Foals Treated with Piperazine-Carbon Disulfide Complex and Untreated Controls

Foal No.	Age when treated (days)	Pretreatment e.p.g.				Post-treatment e.p.g.			
		One week		Day treated		One week		One month	
		Parascaris	Strongyles	Parascaris	Strongyles	Parascaris	Strongyles	Parascaris	Strongyles
Group 1 (150 mg./kg.)									
3	26	0	90	0	90	0	30	0	210
18	56	0	0	0	0	0	0	0	360
12	64	0	90	0	2,430	0	90	0	450
63	86	0	420	0	840	0	120	0	300
66	86	180	600	1,140	2,940	0	0	0	90*
9	103	1,890	750	7,140	980	0	0	0	300
50	105	450	1,350	600	660	0	30	0	300
56	107	750	1,950	1,530	1,800	0	90	0	240
16	109	60	660	0	600	0	60	0	120
91	116	3,810	1,980	4,590	1,800	0	90	0	450*
8	117	1,260	2,040	2,880	3,240	0	0	0	120
73	119	1,530	1,680	1,380	1,860	0	0	..	.....
60	121	5,580	5,730	5,940	3,740	0	90	0	90
58	126	1,800	2,370	2,670	2,340	0	90	..	.....
Total	....	17,310	19,710	27,870	23,320	0	690	0	3,030
Mean	96	1,236	1,408	1,991	1,666	0	50	0	252
Group 2 (100 mg./kg.)									
71	39	0	30	0	0	0	0	0	90
112	51	0	0	30	0	0	0	0	180
82	69	0	30	0	660	0	30	0	330
75	112	30	300	0	390	0	0	0	60
5	94	0	540	960	930	0	0	0	450
61	86	0	900	90	1,830	0	120	..	.....
1	118	180	480	390	930	0	60	0	90*
51	110	690	330	2,430	1,140	0	0	0	150
87S	112	390	2,010	1,680	5,610	0	60	0	60
55	115	750	1,230	660	3,000	0	30	0	210
53	118	330	1,320	840	3,300	0	60	0	120*
152	120	30	1,020	330	2,190	0	210	0	480
87L	123	570	4,830	840	5,430	0	210	0	210
15	124	60	2,820	0	1,470	0	240	0	450*
Total	....	3,030	15,840	8,250	26,880	0	1,020	0	2,880
Mean	99	216	1,131	589	1,920	0	73	0	240
Group 3 (75 mg./kg.)									
220	33	0	90	0	150	0	30	0	60
59	62	0	60	0	150	0	0	..	.....
54	77	0	330	0	1,680	0	270	0	210
72	85	0	60	150	810	0	0	0	270
18	104	3,420	2,940	9,780	4,500	0	300	0	870
70	101	90	720	1,080	2,190	0	0	0	150*
64	110	1,170	2,250	2,280	1,530	0	30	0	210
69	115	180	870	540	2,760	0	60	0	60
65	116	240	1,560	420	2,640	0	270	..	.....
77	117	510	1,200	690	1,680	0	120	0	270
67	126	60	540	600	1,950	0	210	..	.....
11	127	3,570	990	6,000	2,130	0	360	0	210
52	127	1,110	1,650	1,230	2,010	0	90	..	.....
160	142	360	150	3,120	780	0	30	0	240*
Total	....	10,710	13,410	25,890	24,960	0	1,770	0	2,550
Mean	103	765	958	1,849	1,783	0	126	0	255
Group 4 (no treatment)									
6	108	660	1,860	2,820	1,800	2,370	2,310	2,010	1,620
78	128	900	2,220	870	2,370	1,950	8,900	2,100	5,280
4	122	270	1,350	3,870	6,090	1,800	2,250	1,590	6,810
68	91	120	90	1,530	480	1,020	990	3,000	4,830
247	48	0	240	0	120	0	210	0	930
Total	....	1,950	5,760	9,090	10,860	7,140	14,660	8,700	19,470
Mean	99	390	1,152	1,818	2,172	1,428	2,932	1,740	3,894
Group 4A (50 mg./kg.)									
78	147	1,950	8,900	2,100	5,280	0	390	0	450
4	136	1,800	2,250	1,590	6,810	0	1,530	0	3,570
68	105	1,020	990	3,000	4,830	0	90	0	120
247	62	0	210	0	930	0	60	0	30
Total	....	4,770	12,350	6,690	17,850	0	2,070	0	4,170
Mean	113	1,193	3,088	1,672	4,463	0	517	0	1,043

* Treated with 12 ml. CS_2 two weeks after treatment with the suspension.

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RESULTS

Larvae obtained from fecal cultures were all species of small strongyles. No larvae of large strongyles or Strongyloides were found in fecal cultures.

Piperazine-Carbon Disulfide Complex-Treated Foals.—Pretreatment and post-treatment egg counts, obtained on foals treated with the suspension and untreated controls, and the average number of eggs for each group are presented (table 1). The most striking change following treatment was the fact that no Parascaris eggs were found in feces collected one week and one month after treatment at levels of 150, 100, 75, and 50 mg./kg. of body weight.

TABLE 2—Parasites Recovered from Foals Following Treatment with Piperazine-Carbon Disulfide and Untreated Controls

Foal No.	Age when treated (days)	Parascaris	Strongyles*	Oxyuris	*G. intestinalis* instar			*G. nasalis* instar			Total bot larvae
					2nd	2nd-3rd	3rd	2nd	2nd-3rd	3rd	
GROUP 1 (150 mg./kg.)											
3	26	0	0	0	0	0	0	0	0	0	0
18	56	0	10	0	1	2	0	0	0	0	3
12	64	1	10	0	0	0	0	0	0	0	0
63	86	3	50	1	6	0	0	0	0	0	6
66	86	3	25	1	9	1	0	0	0	0	10
9	103	45	50	4	8	0	0	0	0	0	8
50	105	13	75	12	6	1	0	0	0	0	7
56	107	27	25	2	2	1	0	0	0	0	3
16	109	0	10	2	1	0	0	0	0	0	1
91	116	109	100	0	8	3	1	1	0	0	13
8	117	36	200	0	0	0	0	0	0	0	0
73	119	6	50	22	4	0	0	0	0	0	4
60	121	31	50	114	7	0	0	0	0	0	7
58	126	18	25	1	0	0	0	0	0	0	0
GROUP 2 (100 mg./kg.)											
71	39	22	0	0	0	0	0	0	0	0	0
112	51	0	0	0	0	0	0	0	0	0	0
82	69	2	10	0	2	0	0	0	0	0	2
75	112	3	10	0	0	0	0	0	0	0	0
5	94	1	50	0	0	0	0	0	0	0	0
61	86	0	10	0	1	2	0	0	0	0	3
1	118	5	25	16	3	1	0	0	0	0	4
51	110	5	25	12	0	0	0	0	0	0	0
87S	112	126	100	0	3	0	0	2	0	0	5
55	115	12	300	2	0	0	0	0	0	0	0
53	118	4	200	26	5	1	4	0	0	0	10
152	120	2	100	24	1	1	0	0	0	0	2
87L	123	127	400	0	0	0	2	0	0	0	2
15	124	0	50	0	17	4	0	0	0	0	21
GROUP 3 (75 mg./kg.)											
220	33	0	10	0	4	0	0	0	0	0	4
59	62	1	10	0	0	0	0	0	0	0	0
54	77	0	0	0	0	0	0	0	0	0	0
72	85	4	25	0	2	0	0	0	0	0	2
13	104	49	50	6	6	1	0	1	0	0	8
70	101	2	100	18	3	9	1	0	0	0	13
64	110	15	25	10	3	6	1	0	0	0	10
69	115	3	25	5	1	1	0	0	0	0	2
65	116	0	0	0	0	0	0	0	0	0	0
77	117	12	10	15	2	2	0	0	0	0	4
67	126	24	10	40	3	0	0	0	0	0	3
11	127	32	50	1	3	4	0	0	0	0	7
52	127	6	100	65	5	0	0	0	0	0	5
160	142	118	25	0	5	16	0	0	0	0	21
GROUP 4 (no treatment)											
6	108	0	0	0	0	0	0	0	0	0	0
78	128	0	1	0	0	0	0	0	0	0	0
4	122	0	0	1	0	0	0	0	0	0	0
68	91	0	0	0	0	0	0	0	0	0	0
247	48	0	0	0	0	0	0	0	0	0	0
GROUP 4A (50 mg./kg.)											
78	147	71	400	0	9	15	4	0	0	0	28
4	136	71	100	31	6	4	5	3	0	0	18
68	105	22	25	0	0	0	0	0	0	0	0
247	62	0	10	0	2	0	0	0	0	0	2

* Estimate of the number of strongyles recovered by screening. All were small strongyles.

TABLE 3—Number of Parascaris and Strongyle Eggs per Gram Feces in Foals Treated with 12 cc. of CS_2 per Animal After a Previous Treatment with Piperazine-Carbon Disulfide

	Pretreatment e.p.g.				Post-treatment e.p.g.			
	One week		Day of treatment		One week		One month	
Foal No.	Parascaris	Strongyles	Parascaris	Strongyles	Parascaris	Strongyles	Parascaris	Strongyles
91	0	90	0	540	0	180	0	450
66	0	0	0	150	0	30	0	90
15	0	240	0	810	0	270	0	450
1	0	60	0	0	0	90	0	90
53	0	60	0	90	0	60	0	120
160	0	30	0	270	0	150	0	240
70	0	0	0	30	0	30	0	150
Total	0	480	0	1,890	0	810	0	1,590
Mean	0	69	0	270	0	116	0	229

The untreated foals continued to pass large numbers of Parascaris eggs. Strongyle egg counts made one week after treatment were considerably lower than pretreatment egg counts. The average number of strongyle eggs per foal for groups 1, 2, and 3 decreased 97 per cent, 96 per cent, and 93 per cent, respectively, from average egg counts made on the day of treatment; even one month after treatment their egg counts remained low. One week after treatment of the 4 foals in group 4A, at the rate of 50 mg./kg. of body weight, the average number of strongyle eggs decreased 88 per cent of the last pretreatment average, but one month after treatment this count increased to a moderately high level.

The number of parasites recovered from the feces of foals treated with the suspension supports the decrease in egg counts. The numbers of parasites which were recovered from each foal following treatment is shown (table 2). Parascaris and small strongyles were recovered from almost every foal which had positive egg counts before treatment, and immature Parascaris organisms and small strongyles were recovered from several of the youngest foals which had negative egg counts before treatment. Adults of Oxyuris organisms were passed by foals in all treated groups, and in several instances the number passed was high. Stomach bots were recovered from the feces passed by foals in all treated groups. Nearly all of the bots were *Gasterophilus intestinalis*, but a few *Gasterophilus nasalis* organisms were recovered. Second instars, second instars in ecdysis, and third instars were found; however, only a few were mature third instars. Only 1 small strongyle and 1 Oxyuris organism were found in feces passed by the untreated foals.

Seven Foals Treated with CS_2 After a Previous Treatment with Piperazine-Carbon Disulfide Complex.—Pretreatment and post-treatment egg counts for the 7 foals treated with CS_2 after a previous treatment with piperazine-carbon disulfide complex, and the data on parasites recovered from their feces, are presented (tables 3, 4). The egg counts made one week before treatment with CS_2 were the same counts made one week after treatment with the suspension. No Parascaris eggs were found in this group before or after treatment with CS_2, and no Parascaris adults were passed in the feces. The average number of strongyle eggs remained approximately the same after treatment as before treat-

TABLE 4—Parasites Recovered from Feces Following Treatment with 12 cc. CS_2 per Animal After a Previous Treatment with Piperazine-Carbon Disulfide

				G. intestinalis instar			*G. nasalis* instar			Total bot larvae
Foal No.	Parascaris	Strongyles	Oxyuris	2nd	2nd-3rd	3rd	2nd	2nd-3rd	3rd	
91	0	0	0	0	0	0	0	0	0	0
66	0	0	0	0	0	0	0	0	0	0
15	0	0	0	1	0	0	0	0	0	1
1	0	0	0	0	0	0	0	0	0	0
53	0	0	1	1	0	0	0	0	0	1
160	0	0	0	1	0	0	0	0	0	1
70	0	0	0	3	0	0	0	0	0	3
Total	0	0	1	6	0	0	0	0	0	0
Mean	0	0	0.1	0.9	0	0	0	0	0	0.9

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TABLE 5—Group Averages of Hematological Observations on Foals Treated with Piperazine-Carbondisulfide Complex and Untreated Controls

Dosage	Group	Erythrocytes (millions/cmm.)	Hemoglobin (Gm. %)	Sedimentation rate (mm./min.) 15	60	120	Hematocrit (%)	Leukocytes (thousands/cmm.)	Heterophils Non-Seg. %	Heterophils Seg. %	Lymphocytes Large %	Lymphocytes Small %	Monocytes (%)	Eosinophils (%)	Basophils (%)
Pretreatment															
150	1	10.85	13.9	5.6	45.6	52.5	39.1	14.44	0.0	48.7	1.5	41.0	4.5	3.6	0.2
100	2	11.11	14.1	4.3	46.3	52.3	40.4	14.17	0.1	48.6	1.6	40.7	4.4	4.4	0.3
75	3	11.20	13.9	2.9	39.9	46.0	40.5	13.55	0.1	53.1	1.1	36.4	4.9	4.2	0.1
50	4A	11.51	14.2	2.6	33.0	42.6	39.5	16.36	2.0	47.6	1.4	36.8	4.1	7.9	0.3
0	4	10.76	12.7	4.6	44.9	52.1	40.3	16.02	0.1	51.6	2.2	36.8	3.6	5.0	0.5
Two days post-treatment															
150	1	11.27	14.8	3.4	37.4	44.9	39.7	14.73	0.1	43.1	1.9	43.4	4.9	6.4	0.1
100	2	11.19	13.9	2.6	29.3	40.7	41.2	14.91	0.6	43.4	2.4	42.4	5.7	4.7	0.0
75	3	11.94	14.4	0.9	15.5	27.8	42.3	12.91	0.4	46.4	2.2	41.4	4.8	4.6	0.1
50	4A	11.26	13.7	2.4	20.4	30.3	39.6	17.70	0.3	48.3	1.6	37.0	2.6	9.5	0.8
0	4	.....	.....	....	.....	.....	.....	.....	....	.....	....	.....	....	....	....
One month post-treatment															
150	1	10.92	14.2	1.7	20.0	31.2	42.0	14.06	0.5	44.5	1.4	41.8	5.4	6.0	0.3
100	2	10.97	13.6	3.5	27.4	37.6	41.3	17.27	0.5	49.7	1.5	38.9	4.3	4.8	0.1
75	3	10.95	14.1	2.4	28.7	38.2	42.1	15.22	0.8	55.5	1.4	31.6	4.2	5.8	0.4
50	4A	10.97	13.9	1.0	25.4	36.9	41.3	15.33	0.4	49.5	1.1	35.8	4.5	8.5	0.1
0	4	11.50	14.4	2.5	31.7	44.0	39.9	16.30	1.9	47.5	1.3	38.5	3.6	6.9	0.2

ment with CS_2, and no adult small strongyles were passed. One Oxyuris adult and 6 second instar *G. intestinalis* were the only parasites recovered from the 7 foals after treatment with CS_2.

The foal (F-6) which was treated with CS_2 had pretreatment egg counts of 2,370 and 2,010 Parascaris and 1,990 and 1,620 strongyle e.p.g. of feces. One week after treatment the number of Parascaris eggs dropped to zero, but three weeks after treatment there were 240 Parascaris e.p.g. Six adult ascarids were recovered from the feces. One week after treatment, the strongyle egg count dropped to approximately one half the pretreatment count. No small strongyles were recovered from the feces. One Oxyuris organism, 3 second instars, 4 second instars in ecdysis, and 6 third instar *G. intestinalis* were recovered.

When the suspension was administered at the rate of 100 mg./kg. of body weight to foal F-6 two weeks after the above treatment with CS_2, 6 adult Parascaris organisms, 9 pinworms, 4 second instar *G. intestinalis*, and 2 third instar *G. intestinalis* were recovered from the feces. No egg counts were made following treatment with the suspension.

Clinical Observations.—No untoward reactions were observed in the foals immediately after dosing with the suspension nor during the 45-hour period in which the feces were collected. The foals acted normally and ate well. Their feces were normal except for a tendency to be somewhat soft in those foals that passed large numbers of Parascaris organisms at one time. However, no diarrhea occurred.

Five of the 7 foals treated with carbon disulfide showed signs of colic with slight muscular weakness during the first few hours following treatment. They staggered slightly when walking and had a few muscular tremors in the shoulder, croup, and hip regions when standing. These signs disappeared in less than ten hours.

Hematology.—The hemograms for the treated groups and untreated group are summarized in table 5. No significant changes occurred in the erythrocytes, hemoglobin, packed cell volumes, leukocytes, or differential counts in any of the groups. There was a tendency, however, for the sedimentation rate to improve after treatment with the suspension as denoted by a decreased rate. A few of the more heavily parasitized foals began to show a slight

increase in the packed cell volume after treatment and expulsion of the worms.

All of the hematological findings remained within normal limits, except a few high leukocyte counts that were associated with either purulent nasal discharge from upper respiratory infection or injuries.

Discussion

The very effective removal of Parascaris organisms from foals treated with the suspension of piperazine-carbon disulfide complex at 50, 75, 100, and 150 mg./kg. of body weight is in agreement with results obtained by others[1,2,4,5] when using this compound in horses. The recovery of immature ascarids from feces following treatment is of importance, since the worms can be removed from foals at an early age. Also, the passing of ascarid eggs could be prevented with strategic treatment. The early removal of ascarids along with early removal of stomach bots should reduce any adverse effects these parasites might have on the host.

The effective removal of small strongyles by suspension at dosage levels of 75, 100, and 150 mg./kg. is in agreement with previous tests.[1,2,4,5] The 50 mg./kg. dosage level did not show sufficient efficacy to be considered as a recommended dosage.

The data indicate that the suspension is effective in the removal of Oxyuris at 75, 100, and 150 mg./kg. of body weight. Although pretreatment data concerning this species are not available, the large numbers of adult Oxyuris organisms recovered from the feces (table 2) indicate high efficacy. Treatment at 50 mg./kg. caused the removal of 31 Oxyuris organisms from 1 foal and none from 3 foals; therefore, it is difficult to assess the efficacy of the suspension at this level. Only 1 Oxyuris organism was recovered from feces passed by animals in the control group, which strongly indicates that the worms passed by the treated foals resulted from the treatment with the suspension. These results obtained on removal of pinworms are in agreement with the observations of Drudge *et al.*,[1,2] who found this suspension to be efficacious against adult pinworms.

Since the data obtained on removal of Gasterophilus larvae are not based on critical studies, it is difficult to assess the efficacy of the suspension on bots; however, the foals treated with the suspension at all four dosage levels passed larvae of several instars, while the control foals passed no bot larvae. The foals treated with CS_2 after a previous treatment with the suspension passed a total of only 6 second instars of *G. intestinalis*. It is probable that these latter larvae had developed to this stage after treatment with the suspension. The fact that no older bots were removed by CS_2 indicates that the previous treatment with the suspension removed most of the bots present in the stomach. Conversely, in foal F-6, the suspension caused the removal of ascarids, pinworms, 4 second instar *G. intestinalis* larvae, and 2 third instar *G. intestinalis* larvae after a previous treatment with CS_2. The third instars removed by the suspension in this case were present in the host when treated with CS_2, but were not removed.

Summary

Piperazine-carbon disulfide complex (Parvex Suspension) was found to be nontoxic to foals when administered at the rate of 150 mg. per kilogram of body weight, and no blood changes were observed following this treatment.

This suspension was shown to have 100 per cent efficacy in the removal of *Parascaris equorum* at dosages of 150, 100, 75, and 50 mg./kg. of body weight. Both mature and immature ascarids were removed.

The suspension given at the rate of 150, 100, and 75 mg./kg. of body weight showed high efficacy (93 to 97%) against small strongyles. The same dosages caused adult Oxyuris organisms to be passed.

Second and third instars of *Gasterophilus intestinalis* and *Gasterophilus nasalis* were effectively removed by the suspension when given at the rate of 150, 100, and 75 mg./kg. of body weight.

Treatment of foals with CS_2 after being treated with the suspension did not remove a significant number of parasites, but treatment with the suspension after a previous treatment with CS_2 resulted in the removal of ascarids, pinworms, and bot larvae.

References

[1] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., and Hutzler, L. B.: Field Studies with Piperazine-Carbon Disulfide Complex Against Parasites of the Horse. J.A.V.M.A., *131*, (Sept. 1, 1957): 231–233.

[2] Drudge, J. H., Leland, S. E., Jr., Wyant, Z. N., Elam, G. W., Smith, C. E., and Dale, E.: Critical Tests with Piperazine-Carbon Disulfide Complex (Parvex) Against

Parasites of the Horse. Am. J. Vet. Res., *18*, (Oct., 1957): 792–797.

[3] Kelley, G. W., Olson, L. S., and Garwood, V.: A Field Evaluation of Ascaricides in Swine. Vet. Med., *51*, (1956): 97.

[4] Poynter, D.: A Comparative Assessment of the Anthelminthic Activity in Horses of Four Piperazine Compounds. Vet. Rec., *68*, (1956): 291–297.

[5] Poynter, D.: Piperazine-1-Carbodithioic Acid as an Anthelminthic Against *Parascaris Equorum* in Horses. Vet. Rec., *68*, (1956): 429–431.

[6] Riedel, B. B., and Larson, E. J.: The Critical Evaluation of a New Swine Anthelminthic Containing Piperazine and Carbon Disulfide-Parvex. J.A.V.M.A., *129*, (Aug. 15, 1956): 156–159.

Western Equine Encephalomyelitis Virus in Tissue Culture and Color Test Titration of Virus and Its Antibodies in Ehrlich Cells

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WESTERN EQUINE ENCEPHALOMYELITIS (WEE) virus has been shown to be cytopathogenic for HeLa cells [5] and to produce plaques in a monolayer of chicken embryo fibroblasts.[1] In the present experiments, WEE virus was studied in cultures in Ehrlich carcinoma cells, HeLa cells, and guinea pig cells. Virus and antibody titrations were readily achieved by the adaptation of the color test [2-4] to the WEE-Ehrlich cell system.

MATERIALS AND METHODS

Cultures of Ehrlich cells and HeLa cells were cultivated in flasks or tubes, using Melnick's medium (0.5% lactalbumin enzymatic hydrolysate and Hank's balanced salt solution) containing 20 per cent normal horse serum. Cells from trypsinized guinea pig kidneys were grown in Melnick's medium containing 5 per cent normal calf serum. Cultures were incubated at 37 C. in stationary tubes placed in a slightly inclined position until the cells covered the lower third of the tubes. After 72 hours, the original medium was replaced by 5 per cent normal horse serum with Melnick's medium containing Earle's solution.

Cultures of both HeLa cells and Ehrlich carcinoma cells were prepared by removing the nutritive medium and adding 0.3 per cent trypsin solution at 36 C. The cells were then dispersed with a serological pipette and centrifuged for eight minutes at 500 r.p.m. The supernatant trypsin solution was removed and the cells were resuspended in the nutritive medium. Cells were counted in a Neubauer chamber, and the desired concentration was transferred to new tubes with the aid of the Cornwall apparatus or by pipetting. Tube cultures of the continuous line cells were seeded with 35,000 Ehrlich cells or 100,000 HeLa cells; 250 units of penicillin, 250 μg. of streptomycin, and 100 units of mycostatin per milliliter were added.

Virus.—A stock of an Argentine strain of WEE virus was used as a 10 per cent phosphate buffer suspension of infected guinea pig brain. Four tubes of each type were used at each virus dilution. Titrations in suckling mice were made by inoculating, intracerebrally, 7-day-old mice with 0.03 cc. of the virus suspension, using 5 animals per dilution.

Neutralization Tests.—The neutralization tests were carried out in cultures of the three types of cells. Equal amounts of virus and immune guinea pig serum were used. Neutralization was considered positive only when the inhibition of cytopathic degeneration was complete. The amount of virus for the neutralization test was 100 t.c.d.$_{50}$, the serum being used at dilutions of 1:5, 1:20, and 1:80 in phosphate buffer solution. Equal parts were mixed and kept at room temperature for 30 minutes; 0.1 cc. of each mixture was inoculated into each tube.

Color Test.—The color test was the same as the one described by Melnick and Opton,[2] except that Ehrlich cells were used. Cell suspensions were prepared by application of 1:5,000 versene solution. The color test was carried out in the following manner: 140,000 Ehrlich cells in 0.2 ml. of medium were introduced into each tube which already contained the virus-serum mixture, the virus titration, or the serum control. All tubes were covered with 0.9 ml. of sterile liquid petrolatum and incubated at 37 C. The test was read for five to seven days. With large tests, we used the "Disposo Tray*" as described.[2]

* Manufactured by Linbro Chemical Co., New Haven, Conn.

TABLE 1—Color Test Titration (Phenol Red) in Versenated Ehrlich Cells

Titration	pH 7.6	7.4	7.2	7.0	6.8	6.6	No. positive
VIRUS DILUTION* (negative logarithm)							
2.0	4	0	0	0	0	0	4/4†
3.0	4	0	0	0	0	0	4/4
4.0	4	0	0	0	0	0	4/4
5.0	1	0	0	0	0	3	1/4
6.0	0	0	0	0	0	4	0/4
7.0	0	0	0	0	0	4	0/4
ANTIBODY DILUTIONS** (reciprocal)							
2	0	0	0	0	0	4	0/4
4	0	0	0	0	0	4	0/4
8	0	0	0	0	0	4	0/4
16	0	0	0	1	1	2	2/4
32	0	0	0	0	0	0	0/0
64	0	1	2	0	0	1	3/4
128	0	2	1	0	0	1	3/4
256	2	1	0	0	0	1	3/4
512	3	1	0	0	0	0	4/4

* 0.2 ml. virus + 0.2 ml. cells. †Numerator = No. of positive tubes; denominator = No. of tubes inoculated.
** 0.2 ml. serum + 0.2 ml. 10^{-2} virus + 0.2 ml. cells.

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XUM

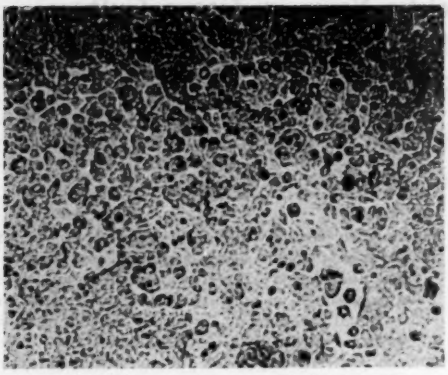

Fig. 1—Monolayer culture of Ehrlich's cells, uninoculated. Osmic acid; x 133.

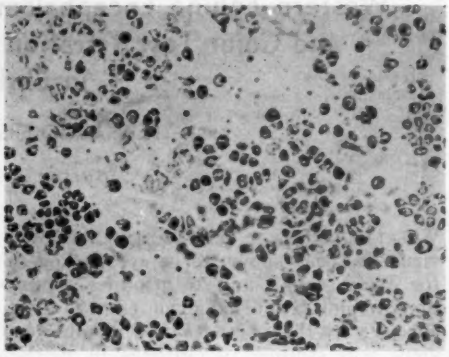

Fig. 2—Ehrlich's cells 18 hours after inoculation with western equine encephalomyelitis virus. Osmic acid; x 133.

RESULTS

The sensitivity of Ehrlich tumor cells to the destructive effects of WEE virus became apparent within 18 to 24 hours. The first change was retraction of the cells (fig. 1, 2), in contrast to cells in uninfected Ehrlich culture; next there was loosening of the cells from the tube walls, and finally complete necrosis. After nine passages in Ehrlich cells, the virus exhibited the same titer (10^5 per ml.) for both Ehrlich and HeLa cells. Cytopathic effects of WEE virus for HeLa cells were seen 24 to 48 hours after addition of virus. In the twelfth passage, titers for guinea pig kidney cells and Ehrlich cells were similar. Cytopathogenic changes resulted in degeneration of the guinea pig kidney cells in 48 hours.

However, there was an increase in titer when tested intracerebrally in mice (10^7 per ml.). At several passage levels, the virus was neutralized by its specific antiserum.

A typical experiment is shown (table 1). The same end points were obtained when titrations were carried out by reading cytopathogenic effects in tube culture monolayers.

DISCUSSION

Ehrlich tumor cells, guinea pig kidney tissue culture, and HeLa cells responded with cytopathogenicity to WEE virus in comparative titrations in tissue cultures and proved lethal when inoculated intracerebrally in mice.

Ehrlich tumor cells offer advantages for the color technique with WEE and perhaps other viruses. They are easy to culture *in vitro* and give a larger yield than HeLa cells. They adapt well to the pH color test, using simple inexpensive nutrient medium, and possess a remarkable resistance to spontaneous degeneration in liquid medium.

SUMMARY

Results are reported of a comparative study of responses to western equine encephalomyelitis (WEE) virus of Ehrlich tumor cells, HeLa cells, and guinea pig kidney cells.

Cytopathogenic reaction was observed in comparative titrations in the different types of tissue cultures used with WEE; for the three systems of tissue cultures, the virus was neutralized by its specific antiserum. Ehrlich cells cultured *in vitro* were found to support the propagation of WEE virus. They offered advantages for use in the color test titration of virus and its antibodies.

References

[1] Dulbecco, R., and Vogt, M.: Some Problems of Animal Virology as Studied by the Plaque Technique. Cold Spring Harbor Symposia on Quantitative Biology, *30*, (1953): 273–279.

[2] Melnick, J. L., and Opton, E. M.: Assay of Poliomyelitis Neutralizing Antibody in Disposable Plastic Panels. Bull. W.H.O. 14, (1956): 129–146.

[3] Robertson, H. E., Brunner, K. T., and Syverton, J. T.: Studies on the Propagation *in Vitro* of Poliomyelitis Viruses. VII. Change of pH in HeLa Cell Cultures for Assay of Virus and Antibody. Proc. Soc. Exptl. Biol. & Med., *88*, (1955): 119.

[4] Salk, J., Youngner, J. S., and Ward, E. N.: Use of Color Change of Phenol Red as the Indicator in Titrating Poliomyelitis Virus or Its Antibody in a Tissue Culture System. Am. J. Hyg., *60*, (1954): 214–230.

[5] Scherer, W. F., and Syverton, J. T.: The Viral Range *in Vitro* of a Malignant Human Cell (Strain HeLa, Gey). Am. J. Path., *30*, (1954): 1075–1082.

Studies of the Reflex Control of the Ruminant Stomach with Special Reference to the Eructation Reflex

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THE PHASE of digestion which normally takes place in the forestomach of ruminants is, to a major degree, under the control of the autonomic nervous system. Ruminoreticular motility, rumination, eructation, and even salivation are apparently controlled through nervous reflexes originating partly in the rumen and reticulum.

This study was directed primarily toward eructation and its relationship to bloat. However, the importance of each of the functions mentioned, as well as their possible interdependence, made it desirable that they be studied together.

REVIEW OF LITERATURE

There is a great deal of evidence indicating that ruminoreticular motility, rumination, and eructation are dependent on intact vagus nerves. Although section of either the dorsal [13, 17, 24, 29] or ventral [13, 17, 24, 29] vagal trunk has little apparent effect on these functions, total vagotomy abolishes all three.[4, 13, 17, 24, 29] Stimulation of the cervical vagus distal to a cut or anesthetized section has been shown to increase the rate of ruminoreticular contraction,[18, 19, 20] while stimulation of the intact dorsal thoracic vagus increases both ruminoreticular rate and eructation rate.[15]

There is also strong evidence that ruminoreticular motility, rumination, eructation, and even salivation are governed in part by nervous reflexes originating in the rumen and reticulum. It has been shown that distension of the reticulum increases its rate of contraction,[7, 14, 20] while a rise in intraruminal pressure increases the rates of both ruminal and reticular contraction.[9, 22, 41] There is also evidence that afferent fibers for an eructation reflex are located in the dorsal thoracic vagus.[28]

Rumination (regurgitation) has been elicited by the application of friction, pressure, or traction to a fairly large area of ruminoreticular mucosa.[1, 37] Schalk and Amadon [37] believed the cranial wall of the rumen was the most sensitive to stimulation.

There is general agreement that eructation is normally associated with rumen contraction [6, 12, 41, 43] and that the rate of eructation is increased with an increase in the intraruminal pressure.[9, 22, 41] Fluoroscopy of sheep,[10, 12] as well as visual examination through the open fistula of cattle,[6, 43] indicated that the reticulum was relatively empty and dilated prior to eructation, thus presenting an exposed cardia.

The degree of cardia exposure is evidently quite critical. Weiss [41] found that cattle, positioned so that the cardias were submerged, were less able to eructate. More recently, Dougherty [11] demonstrated the presence of an eructation-inhibition reflex in sheep. This inhibition, which is initiated by the submersion of the cardia with ingesta or fluid, was absent after the application of butacaine sulfate to the area around the cardia.

Dougherty [11] also surgically removed most of the rumen from decerebrate sheep and then clamped off and insufflated the remaining pocket of reticulum, cardia, and anterior dorsal rumen. He found that eructation continued as before, suggesting that an important number of the receptors is located in this area and that these are stimulated by pressure or distension.

Clark and Weiss [6] were the first to observe that salivation rate increased following tactile stimulation around the cardia. These results were supported by the work of Ash and Kay,[1] although the latter found that stimulating a rather large area of the rumen produced the same results. They also observed that salivation rate increased during rumination, even when the rumen and reticulum were empty. Kay and Phillipson [28] reported that salivation rate increased and then decreased as the intraruminal pressure rose, finally stopping altogether at intraruminal pressures of 15 mm. of Hg and above.

Although the existence of these reflexes appears to be well established, little is known about the type of receptors involved. Relatively little is known about the nerve receptors and reflex mechanisms associated with the digestive tract, and it has been especially difficult to ascribe a specific function to the structures that are observed histologically. Receptors sensitive to both distension and contraction have been demonstrated to be present in the cat stomach,[37] and the forestomach

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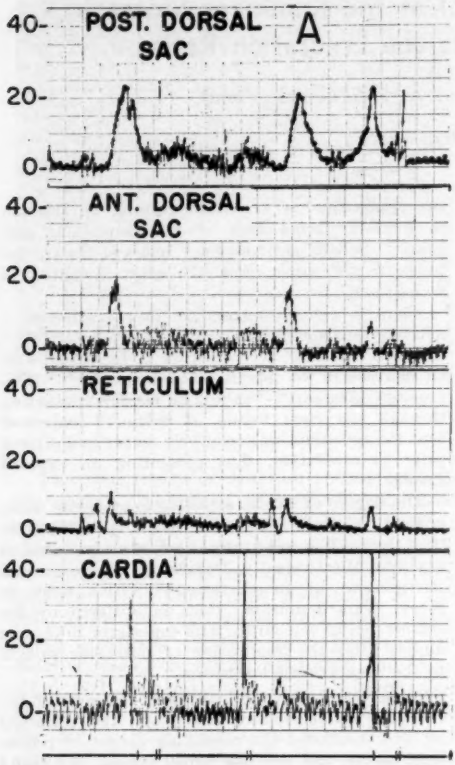
XUM



Graph 1—Record of pressure events (A) in the posterior dorsal sac, anterior dorsal sac, reticulum, and cardia. On the cardia trace, eructation is recorded as a wave having a notch on the ascending limb, deglutition as a more symmetrical wave. Signal marker (bottom line) indicates one mark as an eructation, two marks as a swallow. Notice the occurrence of eructation in conjunction with both primary and secondary rumen contractions (posterior dorsal sac) and also pressure waves on the top three traces simultaneous with eructation. Time line, one large division = 5 sec.; vertical axis, one large division = 5 mm. Hg.

of the goat and sheep.[25, 26] Iggo isolated five vagal fibers in sheep and five in the goat which were activated by these stimuli. He further determined that the receptors were located near the esophageal groove (sheep) or in the anterior wall of the reticulum (goat). Although volume receptors (receptors stimulated by distension but not by active contraction) are described for the simple stomach,[27, 28] Iggo found none of this type in his study of the goat and sheep.

Histological examination of either the simple stomach or the rumen and reticulum provides little evidence for the existence of classic pressure or tactile receptors, and no evidence that tension receptors of the tendon or muscle spindle type

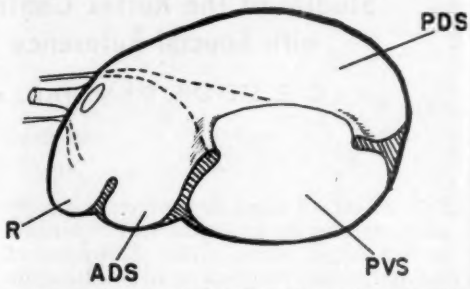


Fig. 1—The drawing represents a diagrammatic cross-section of the posterior esophagus, reticulum, and rumen. Dotted lines represent some of the ruminoreticular branches of the abdominal vagi. Nerves supplying the cardia and anterior rumen are not indicated.

exist.[9, 17, 22, 23, 40] A number of workers have traced fine fibers to the epithelial cells,[16, 17, 22] and there is repeated reference to fine terminal networks of fibers in the muscle of both the simple and ruminant stomach.[9, 17, 22] Habel [17] pointed out that these may be sensitive to pressure or tension. Recently, Hill [22] described a fine network of beaded fibers in the subepithelial connective tissue of the goat rumen. He found these in the region of the posterior pillar and ruminoreticular fold.

Most of the present information on visceral-visceral reflexes is the result of studies involving the cardiovascular and respiratory systems.[2, 3] In the cardiovascular system and, more specifically, in the heart, there is ample evidence that some of the receptors present are sensitive to pressure or tension, while others are sensitive only to distension (volume changes).[20, 21, 34] It has been suggested that the volume receptors may be the nerve networks or arborization first described by Nonidez.[23] Mitchel [30] states that fibril nets are commonly seen in the cardiovascular muscle. In the heart, their sensory nature is indicated by their degeneration following division of the vagus distal to the nodose ganglion. Division of the nerve proximal to this ganglion has no effect.[31] Furthermore, beaded nerve fibers are also common in the primate heart.[41] It would appear, therefore, that the problem of characterizing ruminoreticular receptors is not one of finding structures which could act as receptors, but of assigning functions to the structures already described.

MATERIALS AND METHODS

For the purpose of these experiments, 12 dairy cows were each prepared surgically with a rumen fistula. All of the following experiments were carried out on the unanesthetized animal. Simultaneous pressure recordings were obtained electronically* from the posterior dorsal sac of the

* Four-channel hot-wire recorder manufactured by the Sanborn Co., Cambridge, Mass. Unbonded strain gauges, 0–75 cm. Hg pressure range, manufactured by Statham, Hato Rey, Puerto Rico.

rumen, the anterior dorsal sac of the rumen, the reticulum, and the esophagus (approximately 5 cm. anterior to the cardia). The specific method of recording has been described by Dziuk and Sellers.[10] In a typical control tracing (graph 1), eructation is recorded at the cardia as a rapid wave with a notch on its ascending portion, while swallowing does not show this. This distinction was previously reported by Dziuk and Sellers.[10] Eructation and swallowing were further distinguished from each other by placing a microphone over the pharynx and amplifying the sounds produced. It was hoped that the rate of swallowing might give some indication of the rate of salivation.

The fistula was closed during the experiment by means of a pneumatic plug and, during ruminoreticulum insufflation, nitrogen gas was monitored through a flow meter.

All measurements of rate and amplitude, unless otherwise stipulated, were determined by averaging the values obtained over a five-minute period. Insufflation was always continued for 15 minutes, or until the intraruminal baseline pressure remained relatively constant for 5 minutes or reached 40 mm. of Hg.

Definition of the terms used in the results and discussion are as follows: Ruminoreticular cycle—the period from the beginning of one double contraction of the reticulum until the next. Primary rumen contraction—the first rumen contraction following reticular contraction (actually recorded from and refers to the first posterior dorsal sac contraction). Secondary rumen contraction—all rumen contractions not defined by the previous term (actually recorded from and refers to posterior dorsal sac contractions). Primary eructation—an eructation occurring in conjunction with a primary contraction of the rumen. Secondary eructation—all eructations not defined by the previous term. In all cases, except where stipulated, these occurred in conjunction with secondary rumen contractions. This classification of eructation into primaries and secondaries was done to designate the stage of the ruminoreticular cycle at which each occurs. It is not meant to imply any difference in the character of eructation.

Procedures and Results

Normal Values for Ruminoreticular Motility, Eructation, and Swallowing.—In an initial series of studies, normal values for the rate of eructation, swallowing, ruminoreticular cycles, and secondary rumen contraction, and for the amplitude of posterior dorsal sac contractions were obtained from 10 adult fistulated animals. Nine were grade Holstein-Friesian cows, and 1 (1662) was a grade Jersey cow. For each observation, the animal was prepared for the recording of pressure events, leaving all of the original ingesta in the reticulum and rumen. A minimum period of 30 minutes was allowed for the animal to recover from the various manipulations, and this was then followed by a 15-minute recording period.

The results obtained from this study of the normal ruminoreticular events (table 1) indicate that *(a)* a ruminoreticular cycle occurred approximately once per minute and that approximately one half of these cycles contained a secondary ruminal contraction; *(b)* eructation occurred in conjunction with approximately two thirds of the secondary rumen contractions and 20 per cent of the primary contractions; and *(c)* the ratio of primary to secondary eructations differed markedly between animals, but in each case the rate of secondary eructation was as great or greater than the rate of primary eructation, with a mean ratio of 2 to 1.

After the completion of the above series, a variety of experimental procedures was employed in an attempt to localize ruminoreticular receptors. In addition, an attempt was made in some of the procedures to characterize the receptors involved.

TABLE 1—Normal Values for Pressure Events in 10 Adult Fistulated Dairy Cows

Animal No.	No. of Obs.*	Eructation rate/min.			Rumen rate/min.			Rumen amp.† (mm. Hg)	Ruminoreticular cycles/min.	Swallowing rate/min.
		Total	Prim.	Sec.	Total	Prim.	Sec.			
883	4	0.6	0.3	0.3	1.9	1.2	0.7	7.0	1.2	1.7
[illegible]	7	0.5	0.1	0.4	1.7	1.2	0.5	5.4	1.2	1.3
1467	5	0.5	0.0	0.5	1.8	1.1	0.7	12.0	1.1	1.3
1462	11	0.7	0.2	0.5	1.6	1.0	0.6	8.2	1.0	1.2
1468	2	0.6	0.2	0.4	1.7	1.1	0.6	9.7	1.1	1.5
1510	2	0.5	0.0	0.5	1.6	1.1	0.5	5.0	1.3	1.0
1511	2	0.5	0.2	0.3	1.2	0.9	0.3	5.0	0.9	1.5
1002	2	0.6	0.3	0.3	1.6	0.9	0.7	7.5	0.9	1.6
1611	2	0.8	0.2	0.6	1.8	0.9	0.9	11.0	0.9	0.9
1662	2	0.7	0.2	0.5	1.9	1.1	0.8	10.0	1.1	1.1
Mean**		0.6 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	1.7 ± 0.2	1.0 ± 0.2	0.6 ± 0.2	8.2 ± 2.4	1.1 ± 0.2	1.3 ± 0.3

The values for each animal represent the average value determined from separate observations.
* Each observation represents a measurement over a 5- to 15-minute period; each observation on a given animal was made on a different day. † Amplitude of posterior dorsal sac contraction. ** Mean values for the 10 animals and standard deviation from the mean.

XUM

Receptor and Reflex Arc Localization by the Stimulation and Procainization of Abdominal Vagal Nerves.—Three adult fistulated cows were equipped surgically with electrode assemblies. The assemblies were similar to those used in previous studies[10,11] but were applied to the abdominal vagi; one to the ventral abdominal vagal trunk immediately posterior to the diaphragm and the other two in tandem to the dorsal abdominal vagus. The latter two were attached to the nerve dorsal to the cardia and 10 cm. posterior to this on the ruminal branches. As in previous work,[11] the addition of an empty polyethylene tubing to the electrode assembly allowed for procainization of the included nerve section. Although the surgical procedure was attempted on 3 cows, electrodes remained successfully attached in only 1 of these animals.

When the abdominal vagal nerves of the unanesthetized cow were stimulated by means of attached electrodes, it appeared that stimulation of the ventral vagal trunk increased the rate of secondary rumen contraction and initiated rumination, but had no effect on eructation rate. Stimulation of the dorsal abdominal vagal trunk at the point of attachment of the anterior electrode markedly increased eructation and rumen rates. Stimulation of the main ruminal branches (the point of posterior dorsal electrode attachment) also appeared to increase both eructation and rumen rates, although not as markedly as did the stimulation anterior to this point.

Procainization of the ventral vagal trunk, by means of the electrode assembly, resulted in an atonic reticulum, while procainization of the dorsal trunk appeared to markedly decrease the amplitude of rumen contraction. Stimulation of the dorsal trunk by means of the anterior electrode, after injecting procaine onto the main ruminal branches, resulted in an increase in eructation rate as great or greater than that seen prior to the procainization. This would indicate that afferent fibers were being stimulated.

Receptor Localization and Characterization by the Stimulation of Ruminoreticular Mucosa.—In a series of 35 experiments conducted on 5 cows, an attempt was made to locate and characterize receptors in the rumen and reticulum by the application of stimuli to prescribed areas of the mucosal surface. The stimuli included electrical current, brushing, a water spray, pressure, and weights.

The results of these procedures were often difficult to reproduce. There were, however, certain fairly consistent findings as follows: (1) Simulated tactile stimulation of the cardia area (brush or water spray) increased the rate of ruminoreticular contraction, secondary rumen contraction, eructation, and swallowing; and (2) a local increase in pressure or tension (locally applied pressure, lead weights in the compartments, or weighted saddles placed across the pillars or folds) increased eructation rate when applied to the ruminoreticular fold, anterior pillars, or ventral portion of the anterior dorsal sac. The same stimulus increased the rate of ruminal contractions and possibly the ruminoreticular rate when applied to the areas mentioned or to the ventral portion of the reticulum.

Receptor Localization by the Application of Topical Anesthetics.—In the third series of experiments, topical anesthetics were applied to the mucosa of the rumen and reticulum. The anesthetics used were butacaine sulfate (5–10% solution) and cocaine hydrochloride (5–10% suspension in beeswax). These were applied to specified areas in a series of ten experiments conducted on 5 animals.

The results from this experimental series indicated the following: (1) Butacaine sulfate applied to the cardia area or to the posterior one third of the esophagus decreased the rate of swallowing. (2) The application of either butacaine sulfate or cocaine hydrochloride to an area including the cardia and the dorsal walls of the rumen inhibited eructation and the rate and amplitude of ruminal contraction. (3) Neither anesthetic agent appeared to be effective when applied only to the posterior dorsal sac wall.

Receptor Localization by Procaine Infiltration of Specific Areas of the Rumen and Reticulum.—The results of the previous three experimental series suggested the following procedure in which specified areas of the rumen and reticulum were anesthetized by the infiltration of procaine hydrochloride. A solution of 4 per cent procaine hydrochloride containing 2.5 turbidity reducing units of hyaluronidase* per milliliter was injected by means of a ½-inch,

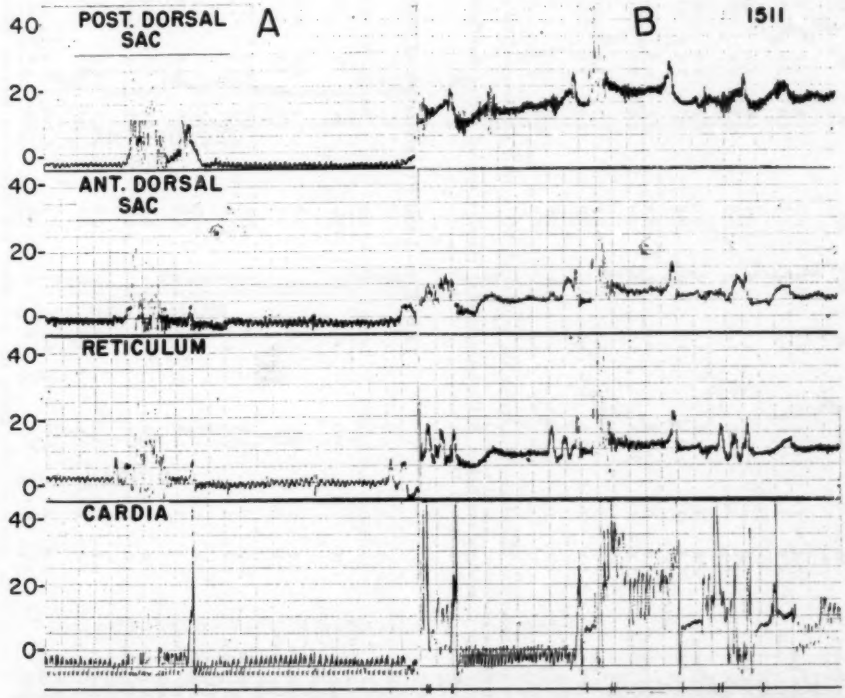
* Pemdase, Pitman-Moore Co., Indianapolis, Ind.

24-gauge needle attached to a syringe by a 3-ft. extension of polyethylene tubing. The needle and polyethylene extension were then taken into the ruminoreticular compartments through the fistula, while the syringe was handled outside of the animal. The injections were made as the needle was directed into and then through the wall from the mucosal surface. The pattern and location of the injection are described later. These experiments were carried out after the other experiments described for these particular animals, because it was feared that infections would result. However, no functional disturbances were noticed following these experiments, and at necropsy there were no observed lesions due to these injections.

In the first series of experiments, the injection was made in a line approximately 10 cm. ventral to the cardia in a horizontal plane across the anterior wall of the reticulum. This was done in an attempt to anesthetize the reticular branches of the ventral vagal trunk. The criterion for an effective nerve block was the absence or marked decrease in amplitude of reticular contractions. Typical records of the pressure events occurring before (graph 2) and after (graph 3) procainization of this area indicate the effect of this procedure on the 5 animals tested (table 2). Nitrogen was insufflated at the same rate before and after procainization.

It would appear that although reticular contraction was inhibited or prevented, rumen contraction, eructation, and swallowing were unaffected, except in cow 1462. Her eructation rate was increased following the injection, and the intraruminal pressure was decreased.

In the second series of experiments, the injection was made into the dorsomedial wall of the anterior dorsal sac in an attempt to block the ruminal branches of the dorsal vagus as they pass over the parietal surface in that area. The injection was made in a line approximately 25 cm. long, perpen-



Graph 2—Control records taken before (A) and during (B) insufflation at 15 liters N_2/min. Rapid oscillations on all traces are due to animal movement. Notice the amplitude of rumen and reticular contraction for comparison to figures 3 and 6 recorded the same day from the same animal (animal 1511, tables 2 and 4).

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TABLE 2—Local Procainization of the Reticular Wall

Animal No. & procedure*	Eructation rate/min.			Swallowing rate/min.			Rumen rate/min.			Rumen amp. (mm. Hg)			Ruminoreticular cycles/min.			Maximum intraruminal pressure (mm. Hg)†		
	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.
1467																		
No gas	0.4	1.0	+0.6	0.8	0.8	0.0	1.6	1.6	0.0	7	6	−1	1.0	1.2	+0.2	....	....	.....
10 l./min.	1.2	1.3	+0.1	1.0	0.3	−0.7	3.0	2.5	−0.5	6	5	−1	1.4	1.2	−0.2	25	22	−3
1662																		
No gas	1.0	0.7	−0.3	1.0	0.6	−0.4	1.6	1.1	−0.5	8	8	0	1.2	....	.....	....	....	.....
5 l./min.	1.1	1.2	+0.1	1.0	1.0	0.0	2.3	2.8	+0.5	6	6	0	1.3	....	.....	26	25	−1
1131																		
3 l./min.	1.3	1.3	0.0	1.0	1.0	0.0	1.2	2.0	+0.8	5	5	0	1.0	....	.....	....	....	.....
3 l./min.	1.3	1.2	−0.1	1.0	1.0	0.0	1.2	2.4	+1.2	5	5	0	1.0	....	.....	13	15	+2
1511																		
No gas	0.6	0.5	−0.1	0.9	1.1	+0.2	1.2	0.9	−0.3	5	7	+2	0.6	0.6	0.0	....	....	.....
15 l./min.	1.8	1.5	−0.3	1.2	1.2	0.0	2.3	2.0	−0.3	5	5	0	1.2	1.0	0.0	30	25	−5
1462																		
No gas	0.6	1.1	+0.5	1.2	1.6	+0.4	0.9	1.4	+0.5	6	5	−1	....	0.0	.....	....	....	.....
15 l./min.	1.2	1.4	+0.2	1.2	1.2	0.0	2.6	1.6	−1.0	7	6	−1	1.4	0.0	−1.4	35	17	−18
Mean																		
No gas	0.6	0.8	+0.2**	1.0	1.0	0.0	1.3	1.3	0.0	7	7	0.0	0.9	....	.....	....	....	.....
Gas	1.3	1.3	0.0	1.1	0.9	−0.2	2.3	2.3	0.0	6	6	0.0	1.2	....	.....	26	21	−5

B = before the procedure; D = during the procedure; Dif. = difference between the values obtained before the procedure (local procainization of the reticular wall) and during the procedure; l. = liter.

* Rate of nitrogen insufflation. † Maximum baseline pressure reached during insufflation, as measured in the posterior dorsal sac of the rumen. ** Mean values before insufflation, excluding animal 1131.

TABLE 3—Local Procainization of Medial Wall of Anterior Dorsal Sac

Animal No. & procedure*	Eructation rate/min.			Swallowing rate/min.			Rumen rate/min.			Rumen amp. (mm. Hg)			Ruminoreticular cycles/min.			Maximum intraruminal pressure (mm. Hg)†		
	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.
1467**																		
No gas	0.4	0.0	−0.4	1.6	1.0	−0.6	2.4	0.0	−2.4	5	0	−5	0.8	0.8	0.0	....	....	.....
10 l./min.	1.6	0.6	−1.0	1.1	1.8	+0.7	2.0	1.4	−0.6	5	5	0	0.7	1.0	+0.3	15	45	+30
1662																		
No gas	1.0	0.0	−1.0	1.0	0.8	−0.2	1.6	0.8	−0.8	8	4	−4	1.2	....	.....	....	....	.....
5 l./min.	1.1	1.5	+0.4	1.0	0.9	−0.1	2.0	2.2	+0.2	5	4	−1	1.0	....	.....	26	40	+14
1511‡																		
No gas	1.0	0.0	−1.0	1.4	1.4	0.0	1.0	0.0	−1.0	3	0	−3	0.8	1.0	+0.7	....	....	.....
15 l./min.	2.6	1.0	−1.6	1.4	1.4	0.0	2.0	0.0	−2.0	5	0	−5	....	....	.....	35	45	+10
1462§																		
No gas	1.0	0.3	−0.7	1.2	1.2	0.0	1.4	0.6	−0.8	7	2	−5	1.1	1.0	−0.1	....	....	.....
15 l./min.	1.5	0.6	−0.9	1.1	1.1	0.0	1.8	1.1	−0.7	7	4	−3	1.4	1.1	−0.3	19	38	+19
1131																		
No gas	0.4	0.0	−0.4	1.0	1.0	0.0	1.2	0.0	−1.2	5	0	−5	0.8	0.8	0.0	....	....	.....
3 l./min.	1.3	0.8	−0.5	1.0	1.0	0.0	2.4	0.0	−2.4	5	2	−3	0.8	....	.....	13	25	+12
Mean																		
No gas	0.8	0.1	−0.7‖	1.2	1.1	−0.1	1.5	0.3	−1.3	6	1	−5	1.0	....	.....	....	....	.....
Gas	1.6	0.1	−1.5	1.1	1.2	+0.1	2.0	0.9	−1.1	5	3	−2	0.9	....	.....	22	39	+17

B = before the procedure; D = during the procedure; Dif. = difference between the values obtained before the procedure and during the procedure; l. = liter.

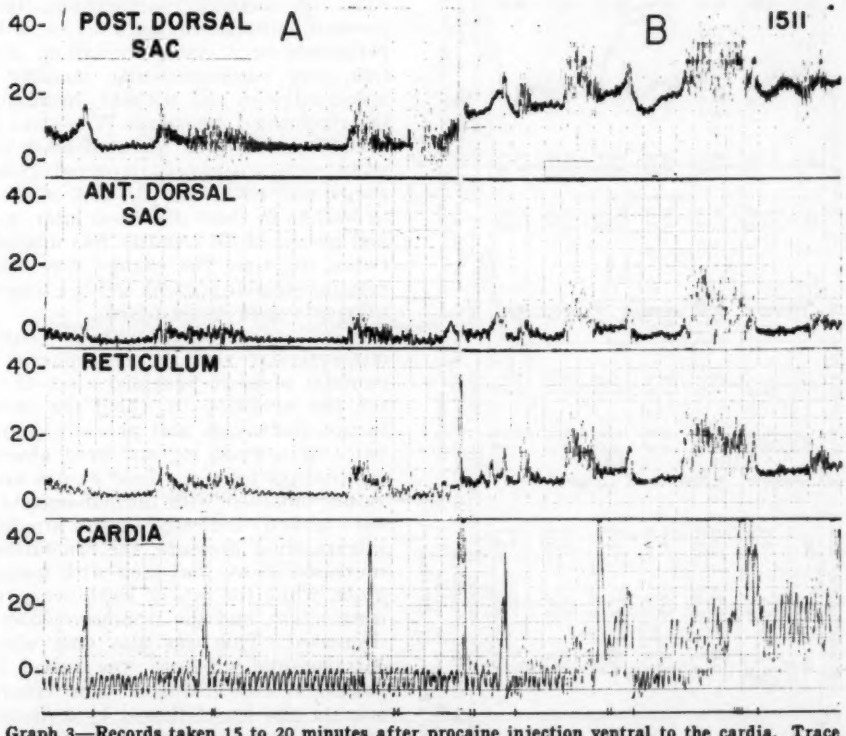
* Rate of nitrogen insufflation. † Maximum baseline pressure reached during insufflation, as measured in the posterior dorsal sac of the rumen. ** Half of the ingesta was removed from the rumen and reticulum prior to the experiment. ‡ All of the ingesta was removed from the rumen and reticulum prior to the experiment. § Figures represent the mean values of five experiments carried out on this animal. ‖ Significant ($P < 0.05$) difference.

dicular to and bisecting an imaginary line drawn between the cardia and the medial border of the anterior ruminal pillar. The criterion of an effective block was the cessation of all contraction of the anterior pillar and the area of the rumen posterior to this, as determined visually. At this time, the rate and amplitude of reticular contraction were unaffected. This was in marked contrast to the effect of blocking the anterior wall of the reticulum.

This procedure, for the most part, stopped eructation until insufflation was started (graphs 4, 5, and table 3) Even during insufflation, with a single exception, the eructation rate was considerably less than that seen prior to procainization. In all 5 cows, intraruminal pressure during insufflation was much higher following the injection of procaine.

In these procainization trials, only enough ingesta were removed to allow for the injection of the procaine, with two exceptions. Before the experiment, approximately one half of the ingesta was removed from the rumen and reticulum of cow 1467, and all of the ingesta was removed from cow 1511. Although it was realized that this introduced another variable to the comparison of animals (the preinjection normals were also taken after ingesta removal), it did not appear to greatly influence the effect of the procedure. Furthermore, it lent support to the proposition that the decrease in eructation rate seen in this particular series cannot be satisfactorily explained by submersion of the cardia alone. Otherwise, the effect of this procedure on eructation might be explained by the inability to expose the cardia as a result of anesthetizing the anterior pillar.

In a third series of experiments, the procaine injection was made in a hemielliptical line into the ventral one half of the cardia



Graph 3—Records taken 15 to 20 minutes after procaine injection ventral to the cardia. Trace A was taken before and B during insufflation at 10 liters N_2/min. Notice reticular contractions near right edge of trace A and left edge of trace B, showing a very small amplitude. Rumen contractions are essentially the same as those in the control trace (graph 2). Four marks (bottom line) designate urination or defecation (animal 1511, table 2).

was removed from the rumen and reticulum prior to the experiment. ‡ All of the ingesta was removed from the rumen and reticulum prior to the experiment. § Figures represent the mean values of dye experiments carried out on this animal. ‖ Significant ($P < 0.05$) difference.

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TABLE 4—Local Procainization of the Cardiac Sphincter

Animal No. & procedure*	Eructation rate/min.			Swallowing rate/min.			Rumen rate/min.			Rumen amp. (mm. Hg)			Ruminoreticular cycles/min.			Maximum intraruminal pressure (mm. Hg)†		
	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.
1467																		
No gas	0.3	0.1	−0.2	0.8	1.0	+0.2	1.7	....	.....	6	..	....	0.9	....	.....	....	....	.....
15 l./min.	1.1	1.7	+0.6	0.9	0.3	−0.6	2.2	2.0	−0.2	6	6	0	1.2	....	.....	45	20	−25
1662																		
No gas	0.6	1.2	+0.6	1.3	1.0	−0.3	1.5	2.8	+1.3	7	3	−4	0.9	....	.....	....	....	.....
5 l./min.	1.0	2.3	+1.3	1.2	0.2	−1.0	3.1	3.0	−0.1	5	3	−2	1.5	....	.....	33	10	−23
1131																		
No gas	1.0	1.0	0.0	1.8	1.2	−0.6	1.8	1.2	−0.6	2	2	0	0.8	0.8	0.0	....	....	.....
5 l./min.	1.0	1.8	+0.8	2.3	1.4	−0.9	3.0	2.4	−0.6	4	5	+1	....	....	.....	20	15	−5
1462																		
No gas	0.6	1.1	+0.5	1.2	1.1	−0.1	1.6	1.3	−0.3	10	11	+1	1.0	0.8	−0.2	....	....	.....
15 l./min.	1.3	2.0	+0.7	0.8	0.8	0.0	2.3	1.8	−0.5	10	8	−2	1.2	1.1	−01	45	35	−10
1511																		
No gas	0.5	0.5	0.0	1.0	0.8	−0.2	1.1	2.2	+1.1	5	5	0	0.6	....	.....	....	....	.....
15 l./min.	1.5	6.7	+5.2	1.0	1.0	0.0	2.2	2.3	+0.1	5	5	0	1.2	....	.....	44	17	−27
Mean																		
No gas	0.6	0.8	+0.2	1.2	1.0	−0.2	1.7	1.9	+0.2	6	4	−2	0.9	....	.....	....	....	.....
Gas	1.2	2.9	+1.7**	1.2	0.7	−0.5	2.7	2.4	−0.3	6	5	−1	1.3	....	.....	37	19	−18

B = before the procedure; D = during the procedure; Dif. = difference between the values obtained before the procedure and during the procedure; l. = liter.
* Rate of nitrogen insufflation. † Maximum baseline pressure reached during insufflation, as measured in the posterior dorsal sac of the rumen. ** The average is high, due to the high value for animal 1511, but if the value for that animal is discarded, the mean value for the remaining 4 animals during insufflation is +0.8 and still represents a significant ($P < 0.05$) difference.

in an attempt to block part of the cardiac sphincter area without blocking the dorsal abdominal vagus. Recordings from cow 1511 were taken before (graph 2) and after (graph 6) the injection described and the results from the series of 5 animals were tabulated (table 4). Prior to insufflation, this particular procedure appeared to have no consistent effect. However, insufflation after the injection resulted in a significant increase in the eructation rate as compared with that seen during the insufflation period prior to injection. There was also a concurrent decrease in the intraruminal pressure. It was also noticed that eructation now occurred, at times, dissociated from rumen contraction.

Receptor Localization and Characterization by Variation of Intraruminal Pressures and by Changing the Physical Character of the Rumen and Reticular Contents.—The following series of experiments was designed in an effort to test, separately, the effect of chemical, tactile, and tension-pressure stimulation. In a series of 12 experiments on 7 cows, eructation, deglutition, and ruminoreticular motility were measured with and without insufflation of the rumen and reticulum. The same measurements were then repeated after the ingesta were completely removed from the rumen and reticulum. In this experiment, as well as in those described later, a minimal period of 30 minutes was allowed between the time the change was made in ruminoreticular content and the time measurements were again taken.

Measurements made with an empty ruminoreticular vault at atmospheric intraruminal pressure provided a set of values for the condition in which the chemical, tactile, distension, and pressure characteristics of normal ingesta were absent. A comparison between these values and the values obtained with normal ingesta present (table 5) indicated that at atmospheric intraruminal pressure, the eructation rate increased above that seen with normal ingesta, while the rate of swallowing, rumen contraction, and the ruminoreticular cycle decreased. This was also true when the intraruminal pressure was raised by insufflation with nitrogen. The removal of ingesta also was followed by a decrease in the amplitude of rumen contraction.

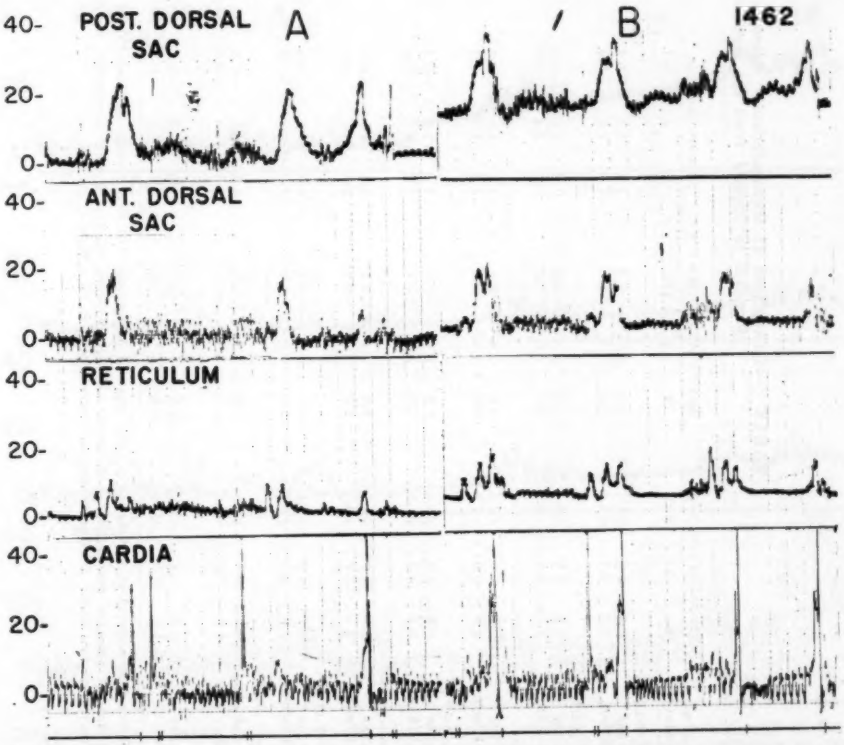
Removal of the ingesta allowed the cardia to be exposed to the gas throughout the ruminoreticular cycle. For that reason, the

increased eructation rate, especially during insufflation of the rumen, could be explained by additional eructations occurring between rumen contractions. However, it was found that only 3 of the 7 animals (883, 1646, 1662) eructated between contractions of the rumen, and 1 of these (1646) did so only at intraruminal pressures below 11 mm. Hg. Also, for a given rate of insufflation, the complete removal of ingesta substantially improved the total eructation efficiency in only 1 of the 7 animals.

In a separate series of experiments on 5 cows, the procedure was expanded in an attempt to separate the effects of chemical, tactile, and pressure-tension stimuli. Measurements were again made with normal ingesta present; then the same animals were subjected to specified variations in the phyical content of the rumen and reticulum and the measurements were repeated. However, in this series the effect of varying the intraruminal pressure was also included with each of the ingesta conditions.

First, a series of recordings was made on each animal with the normal ingesta present. Each record was taken over a five-minute period (at the end of the 30-minute interval mentioned) and the mean values for the events during that period were determined. In this "normal ingesta" series, a total of 70 such determinations were made on the 5 animals, at intraruminal pressures varying between atmospheric pressure and 40 mm. Hg. This procedure was repeated for each of the other three conditions of altered ruminoreticular content, differing only in that the number of determinations varied between 25 and 35 for each series.

After the series of recordings had been made on each animal with the original in-



Graph 4—Control records taken before (A) and during (B) insufflation at 15 liters N_2/min. Notice rumen and reticular contraction rate and amplitude for comparison to graph 5. Also notice eructations in conjunction with primary rumen contractions when animal is bloating in trace B (animal 1462, table 3).

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TABLE 5—Comparison of Ruminoreticular Pressure Events Before and After the Removal of Ingesta from the Reticulum and Rumen

Animal No. & procedure*	Eructation rate/min.			Swallowing rate/min.			Rumen rate/min.			Rumen amp. (mm. Hg)			Ruminoreticular cycles/min.			Maximum intraruminal pressure (mm. Hg)†		
	B	A	Dif.	B	A	Dif.	B	A	Dif.	B	A	Dif.	B	A	Dif.	B	A	Dif.
883 (6-29-56)																		
No gas	0.6	2.0	+1.4	2.0	1.0	−1.0	1.4	1.5	+0.1	7	7	0	0.7	1.0	+0.3	....	....	.....
2 l./min.	2.0	3.4	+1.4	0.6	1.4	+0.8	2.2	1.8	−0.4	10	6	−4	1.5	1.2	−0.3	18	8	−10
1131 (6-27-56)																		
No gas	0.6	0.2	−0.4	1.0	0.4	−0.6	1.8	1.0	−0.8	7	5	−2	1.2	0.7	−0.5	....	....	.....
2 l./min.	1.0	1.0	0.0	0.8	0.5	−0.3	2.4	2.4	0.0	8	6	−2	1.4	0.8	−0.6	10	10	0
1646**																		
No gas	0.6	0.9	+0.3	2.2	1.4	−0.3	1.7	1.5	−0.2	13	4	−9	1.1	0.8	−0.3	....	....	.....
15 l./min.	1.9	3.0	+1.1	2.6	1.3	−1.3	2.2	1.7	−0.5	10	5	−5	1.2	1.0	−0.2	16	26	+13
1884 (8-18 56)																		
No gas	0.4	1.2	+0.8	1.2	0.9	−0.3	1.4	1.1	−0.3	8	3	−5	1.0	0.7	−0.3	....	....	.....
10 l./min.	1.2	3.0	+1.8	1.9	2.0	+0.1	2.7	2.3	−0.4	7	3	−4	1.3	1.0	−0.3	11	15	+4
1511 (3-29-57)																		
No gas	0.5	1.0	+0.5	1.5	1.4	−0.1	1.2	1.0	−0.2	5	3	−2	0.9	0.8	−0.1	....	....	.....
15 l./min.	2.4	2.6	+0.2	2.0	1.4	−0.6	2.4	2.2	−0.1	7	4	−3	1.4	1.0	−0.4	15	18	+3
1662 (7-11-57)																		
No gas	0.7	1.8	+1.1	1.1	0.4	−0.7	1.9	1.4	−0.5	10	5	−5	1.1	1.0	−0.1	....	....	.....
15 l./min.	2.0	3.2	+1.2	1.9	0.0	−1.9	2.0	3.2	+1.2	10	4	−6	1.4	1.4	0.0	27	25	−2
1462‡																		
No gas	0.7	0.8	+0.1	1.2	1.2	0.0	1.6	1.3	−0.3	8	6	−2	1.0	0.8	−0.2	....	....	.....
Gas	1.5	1.3	−0.2	1.1	1.0	−0.1	2.5	1.9	−0.6	9	7	−2	1.3	1.2	−0.1	28	28	0
Mean																		
No gas	0.6	1.1	+0.5	1.5	1.0	−0.5	1.6	1.3	−0.3	8	5	−3	1.0	0.8	−0.2	....	....	.....
Gas	1.7	2.5	+0.8	1.5	1.1	−0.6	2.3	2.2	−0.1	9	5	−4	1.2	1.1	−0.1	18	19	+1

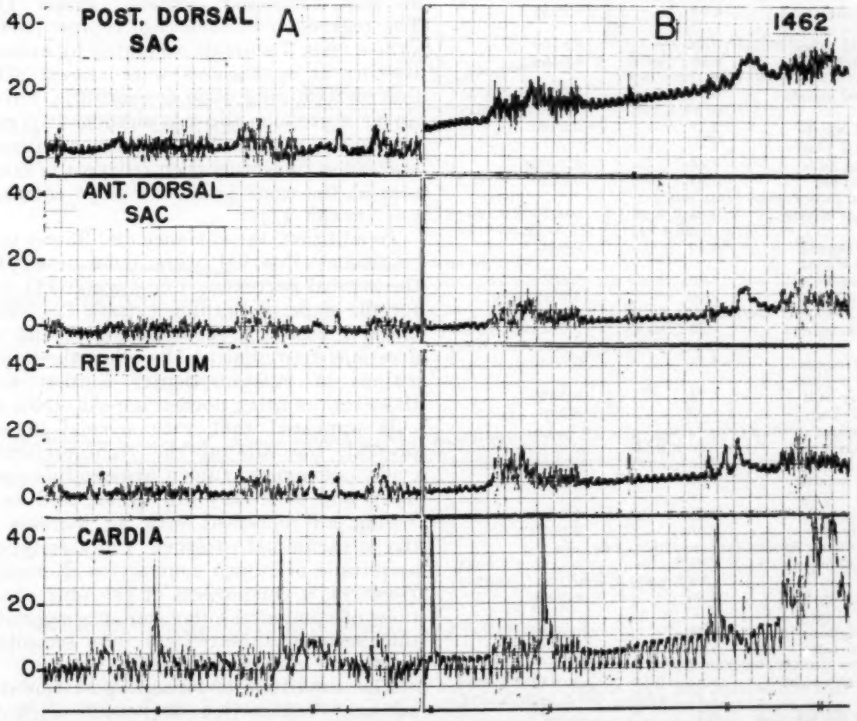
B = before removal of ingesta; A = after removal of ingesta; Dif. = difference between values obtained before and after the removal of ingesta; l. = liter.

* Rate of nitrogen insufflation. † Maximum baseline pressure reached during insufflation, as measured in the posterior dorsal sac of the rumen. ** Mean values for experiments on two days. ‡ Mean values for experiments on four days.

gesta in the rumen and reticulum, these compartments were emptied of their contents and the ingesta were strained free of roughage by means of a sausage press and cheesecloth filter. Water, equivalent in weight to the removed roughage, was added to the fluid portion and the resulting mixture reintroduced into the organ. Again a series of recordings was taken before and during nitrogen insufflation. This "fluid ingesta" closely duplicated normal ingesta in regard to weight and chemical constituents, differing primarily in the decrease in volume (as a contributor to tension stimuli) and the absence of roughage (as a source of tactile stimulation).

Following this, 121 ping-pong balls were added to the fluid ingesta. Prior to the experiment, coarse sand was glued to the surface of each ball in an attempt to provide a tactile stimulus during contraction. They were observed to form a layer about 3 inches deep at the fluid-gas interface, and during ruminoreticular contraction came in contact with the cardia area and most of the dorsolateral and dorsal ruminoreticular wall. The addition of the ping-pong balls was an attempt to provide a source of tactile stimulation, but in addition resulted in an ingesta volume approaching that seen with normal ingesta.

After repeating the recording procedure with the fluid plus ping-pong balls present, the ruminoreticular contents were again removed and a large plastic bag was placed in the posterior ventral sac of the rumen. Fluid was added to the bag until the surface of the fluid was level with the dorsal border of the anterior pillar of the rumen in its uncontracted state. The bag was then closed. A final series of recordings was taken under this condition, in which all



Graph 5—Records taken 10 to 15 minutes after procaine injection into dorsomedial wall of the anterior dorsal sac of the rumen. Trace A was taken before and B during insufflation at 15 liters N_2/min. Notice reticular contractions are essentially normal in rate and amplitude; rate and amplitude of contraction of dorsal sacs of rumen small. No increases in the rates of rumen contraction are seen with increased intraruminal pressure and no eructations as animal starts to bloat (B) (animal 1462, table 3).

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compartments except the posterior ventral sac remained free of fluid or roughage and in which the resting posterior ventral sac contained approximately the same weight of fluid as it had in the series with fluid ingesta. However, the fluid was contained in a plastic bag so that it could no longer move and, in addition, provided considerable resistance to compression. Since the reticulum and anterior dorsal sac were left empty, this condition differed from the previous three in both volume and weight distribution. However, it provided distension of the posterior ventral sac of the rumen and the ruminal pillars, and also provided a resistance to the contraction of these structures. At the same time, it reproduced some of the characteristics of the empty rumen and reticulum (absence of chemical stimulation, relative absence of tactile stimulation, exposed cardia).

Sample recordings (graphs 7, 8, 9) were taken the same day from the same animal under three different conditions of ruminoreticular content; normal ingesta, fluid ingesta, and the condition in which a fluid-filled plastic bag was placed in the posterior ventral sac of the rumen. Also, a recording (graph 10) was taken from this same animal after the complete removal of all rumen and reticular contents.

For the graphic representation of these data, a rectilinear regression line was determined for the effect of intraruminal pressure on each of the measured pressure events, under each of the four conditions of ruminoreticular content (graph 11). The regression in each case represents the values from 5 animals, weighted by animal. Rectilinear regressions were chosen after first determining that a quadratic, curvilinear regression did not significantly represent the relationships. These rectilinear regression lines were then redrawn to intercept at the same point, for slope comparison (graph 12).

In addition, the variance and 95 per cent confidence interval were determined for the normal regression line (graph 11) and for the slope of the line (graph 12). This allowed bases for comparison of the regressions representing the other three conditions of ruminoreticular content with those representing normal ingesta (table 6).

It appeared that with normal ingesta present, the rate of the ruminoreticular cycle, secondary rumen contraction, primary and secondary eructation, and swallowing all increased with an increase in the intraruminal pressure. Conversely, the amplitude of rumen contraction decreased. However, a comparison of the four conditions of ruminoreticular contents suggested that some of these effects were secondary to others.

The amplitude of rumen (posterior dorsal sac) contraction decreased markedly when fluid ingesta were substituted for normal ingesta and increased only slightly when the ping-pong balls were added. However, the presence of a smooth-walled, fluid-filled plastic bag in the posterior ven-

TABLE 6—Slopes and Intercepts for Rectilinear Regressions of Intraruminal Pressure Versus Ruminoreticular Pressure Events

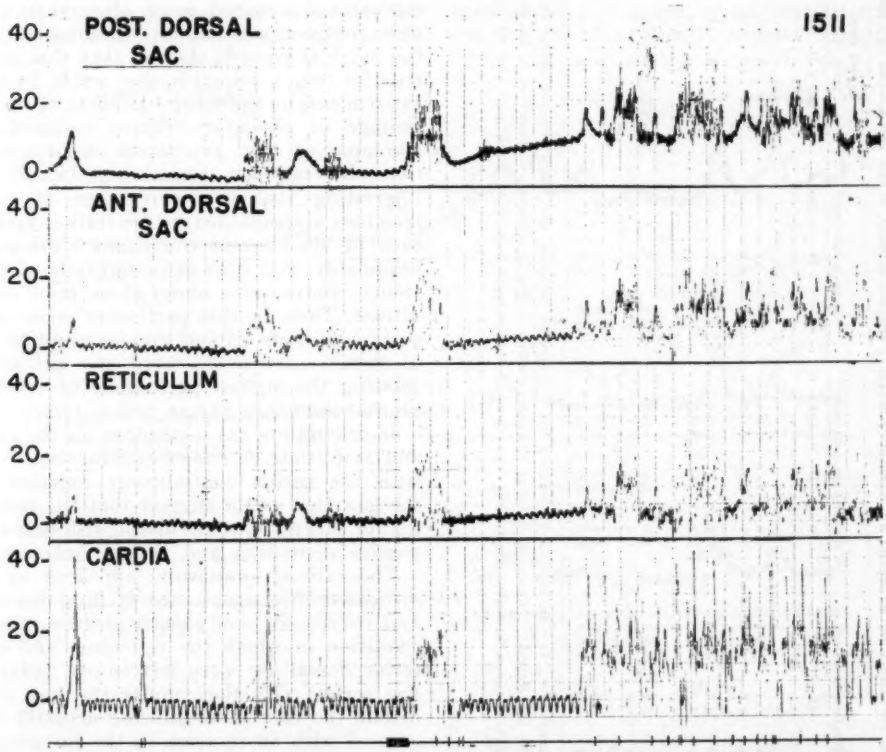
Ingesta	Intercept	Slope
PRIMARY ERUCTATION *(rate/min.)*		
Normal ingesta	0.36 ± 0.11*	0.0215 ± 0.0071
	(0.05, 0.67)†	(0.0021, 0.0409)
Fluid ingesta	0.30	0.0135
Fluid + ping-pong balls	0.44	0.0100
Plastic bag	0.30	0.0100
SECONDARY ERUCTATION *(rate/min.)*		
Normal ingesta	0.53 ± 0.08	0.0221 ± 0.0051
	(0.32, 0.74)	(0.0080, 0.0362)
Fluid ingesta	0.18	0.0280
Fluid + ping-pong balls	0.46	0.0200
Plastic bag	0.74	0.0215
TOTAL ERUCTATION *(rate/min.)*		
Normal ingesta	0.84 ± 0.18	0.0474 ± 0.124
	(0.34, 1.34)	(0.0313, 0.0817)
Fluid ingesta	0.40	0.044
Fluid + ping-pong balls	0.96	0.023
Plastic bag	1.40	0.035
SWALLOWING *(rate/min.)*		
Normal ingesta	1.33 ± 0.09	0.00654 ± 0.0063
	(1.07, 1.59)	(−0.0109, 0.0239)
Fluid ingesta	1.21	0.0000
Fluid + ping-pong balls	1.24	0.0070
Plastic bag	1.09	−0.0080
RUMINORETICULAR *(rate/min.)*		
Normal ingesta	1.12 ± 0.06	0.0149 ± 0.0045
	(0.97, 1.27)	(0.0025, 0.0273)
Fluid ingesta	1.20	0.0085
Fluid + ping-pong balls	1.18	0.0080
Plastic bag	1.06	0.0070
SECONDARY RUMEN CONTRACTION *(rate/min.)*		
Normal ingesta	0.63 ± 0.08	0.0169 ± 0.0053
	(0.40, 0.85)	(0.0023, 0.0315)
Fluid ingesta	0.32	0.0270
Fluid + ping-pong balls	0.44	0.0260
Plastic bag	0.50	0.0250
RUMEN AMPLITUDE *(mm. Hg)*		
Normal ingesta	8.84 ± 0.53	−0.0443 ± 0.0353
	(7.37, 10.31)	(−0.1410, 0.0524)
Fluid ingesta	5.4	0.030
Fluid + ping-pong balls	5.9	0.020
Plastic bag	8.5	−0.065

* Mean value ± one standard deviation. † 95 per cent confidence interval for normal value (all experimental values falling outside of this interval are underlined).

tral sac appeared to give amplitudes close to those of normal ingesta. This indicated that the stimulus was pressure or tension, rather than chemical or tactile.

Both the ruminoreticular cycle and the secondary rumen contractions increased in rate with an increase in the intraruminal pressure (graph 12). The fact that this was true under the four different conditions of ingesta content suggested that tension or pressure was an important stimulus for the reflex control of their respective rates. However, at atmospheric intraruminal pressures, the ruminoreticular cycle appeared to be little affected by the various changes in the content of these organs, while the rate of secondary ruminal contraction was more markedly affected (graph 11). Also, when the intraruminal pressure was increased, the rate of secondary rumen contraction approached the same value, regardless of the ingesta type present. No such compensatory effect was seen in the case of the ruminoreticular cycles when the intraruminal pressure was increased.

The observation that the plastic bag appeared to improve the secondary rumen contractions (at atmospheric intraruminal pressures) as well or better than the addition of ping-pong balls might indicate that the detrimental effect of fluid ingesta was not due to the lack of chemical or tactile stimulation. The difference may have been that the fluid lacked the volume of normal ingesta and, therefore, did not provide the same degree of distension.



Graph 6—Records taken 10 to 15 minutes after injection of procaine into the ventral one half of the cardia. Trace A was taken before and B after insufflation. The beginning of the insufflation period is designated by the prolonged signal (signal marker, bottom line). Notice the reticular contractions appear to be absent, but posterior dorsal sac contractions are present and essentially the same as in control trace (graph 2). Eructation becomes rapid, and is seen to be dissociated from posterior dorsal sac contraction when intraruminal pressure reaches approximately 10 mm. Hg. This result was seen in varying degrees in all 5 of the animals in this series (animal 1511, table 4).

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TABLE 7—Effect of Atropine on Ruminoreticular Pressure Events When the Cardia is Continuously Exposed to Intraruminal Gas

Animal No. & procedure*	Eructation rate/min.			Swallowing rate/min.			Rumen rate/min.			Rumen amp. (mm. Hg)			Ruminoreticular cycles/min.			Maximum intraruminal pressure (mm. Hg)†		
	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.	B	D	Dif.
1646 (7-1-58)**																		
No gas	0.8	0.4	−0.4	1.6	1.3	−0.3	1.8	0.7	−1.1	6	3	−3	1.0	0.0	−1.0	....	....	.....
15 l./min.	5.4	3.6	−1.8	1.7	1.4	−0.3	3.7	2.6	−1.1	5	3	−2	1.1	....	.....	33	45	+12
(7-8-58)‡																		
No gas	1.8	1.2	−0.6	1.4	1.4	0.0	1.6	1.2	−0.4	4	3	−1	0.8	0.8	0.0	....	....	.....
15 l./min.	3.0	4.0	+1.0	1.3	1.6	+0.3	....	....	.....	"	"	....	1.0	....	.....	36	40	+4
1462 (7-3-58)**																		
No gas	1.0	0.2	−0.8	1.6	1.0	−0.6	1.2	1.2	0.0	16	4	−12	0.8	0.0	−0.8	....	....	.....
15 l./min.	1.7	0.6	−1.1	1.5	0.1	−1.4	1.8	1.6	−2.0	14	5	−9	1.1	0.0	−1.1	24	45	+21
(3-26-58)‡																		
No gas	1.8	0.6	−1.2	1.4	1.2	−0.2	1.8	1.0	−0.8	5	2	−3	1.2	0.0	−1.2	....	....	.....
15 l./min.	1.4	0.6	−0.8	1.0	0.6	−0.4	2.6	1.6	−1.0	5	4	−1	1.2	0.0	−1.2	45	40	−5

B = before the procedure; D = during the procedure; Dif. = difference between the values obtained before the procedure and during the procedure; l. = liter.

* Rate of nitrogen insufflation. † Maximum baseline pressure reached during insufflation, as measured in the posterior dorsal sac of the rumen. ** Rumen and reticulum were emptied of all ingesta and a fluid-filled plastic bag was placed in the posterior ventral sac. ‡ Rumen and reticulum were emptied of all ingesta and left empty.

The fact that increasing the intraruminal pressure did not increase the ruminoreticular rate as rapidly under the experimental ingesta conditions as it did when normal ingesta were present indicated some stimulus was lacking. The slopes of the regression lines representing fluid ingesta, fluid ingesta plus ping-pong balls, and the plastic bag were too close together to permit any indication of what that stimulus might be.

As a general rule, eructation appeared to vary directly with the rate of rumen contraction. At atmospheric intraruminal pressure (table 5), the percentage of the secondary rumen contractions accompanied by eructation was less with the fluid ingesta present (56%) than it was with normal ingesta (84%). In the plastic bag series, eructations occurred more often than rumen contractions, but an examination of the original records showed that this held true for only 1 animal in that series. In the experiments on the other 4 animals, the percentage of secondary rumen contactions accompanied by eructation approached that observed for normal ingesta. It is interesting that this percentage of contractions accompanied by eructation under each of the ingesta conditions correlated reasonably well with the amplitude of the rumen contractions under those same conditions. Thus, in this particular series, ingesta conditions giving greater amplitudes of rumen contraction were also the ones showing the highest percentage of eructation on secondary rumen contractions.

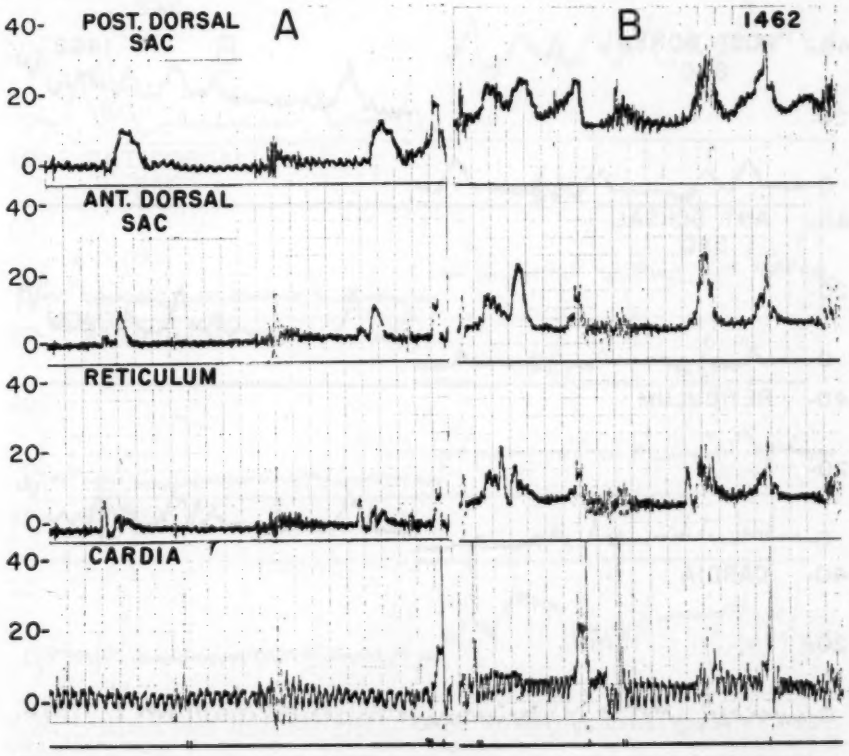
If eructation was dependent on the rate and amplitude of rumen contraction, even with the cardia continuously exposed to the gas, this would suggest that the receptor for this reflex could distinguish between passive distension and active contraction.

The rate of swallowing appeared to be decreased in the presence of fluid ingesta and decreased to a greater degree in the condition in which the reticulum and anterior dorsal sac were left empty (plastic bag series). Further, under the last condition, the rate of swallowing actually decreased with an increase in the intraruminal pressure. If one might assume a correlation between the rate of swallowing and the rate of salivation, this would suggest that pressure or tension alone inhibited (and a tactile stimulus increased) a salivary reflex originating in the anterior rumen or reticulum, or both.

Effect of Atropine on Eructation.—It has been demonstrated by others and in this laboratory that systemic administration of atropine sulfate inhibits ruminoreticular motility and eructation in cattle. In the light of Dougherty's demonstration that eructation may be reflexly inhibited by the submersion of the cardia with ingesta, atropine conceivably could inhibit eructation by preventing exposure of the cardia to the intraruminal gas. To test this possibility, atropine sulfate was administered intramuscularly at doses of 0.4 mg. per pound of body weight. This was done in four experiments on 2 cows. Prior to each experiment, the rumen and reticulum were completely emptied of ingesta. One set of experiments was carried out with both organs completely empty, and another set of experiments was done after the addition of a fluid-filled plastic bag to the posterior ventral sac of the rumen. In either case, the reticulum was left empty and the cardia continuously exposed to the intraruminal gas.

The results of these trials (table 7) indicated that the administration of atropine was followed by a decrease in eructation rate at atmospheric intraruminal pressures and (with a single exception) during insufflation. Also, after atropinization, insufflation resulted in higher intraruminal pressures, again with one exception.

From these results, it would seem that atropine inhibited eructation rate by some means other than by simply preventing exposure of the cardia. Since the effector organ (esophagus) is presumably striated muscle and the dosage was below that known to produce central effects, the atropine apparently acted by preventing contraction of the ruminoreticular muscle.



Graph 7—Records taken before removing normal ingesta from the rumen and reticulum. Trace A was taken before and trace B during insufflation at 10 liters N_2/min. Notice amplitude of reticulum and rumen contractions and rate of eructation for comparison to tracings in graphs 10 and 11, recorded from this same animal the same day (animal 1462, 8-7-56).

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This would again indicate that the initiation of the eructation reflex depends mainly on rumen contraction and the sensitivity of receptors to tension rather than to pressure or touch.

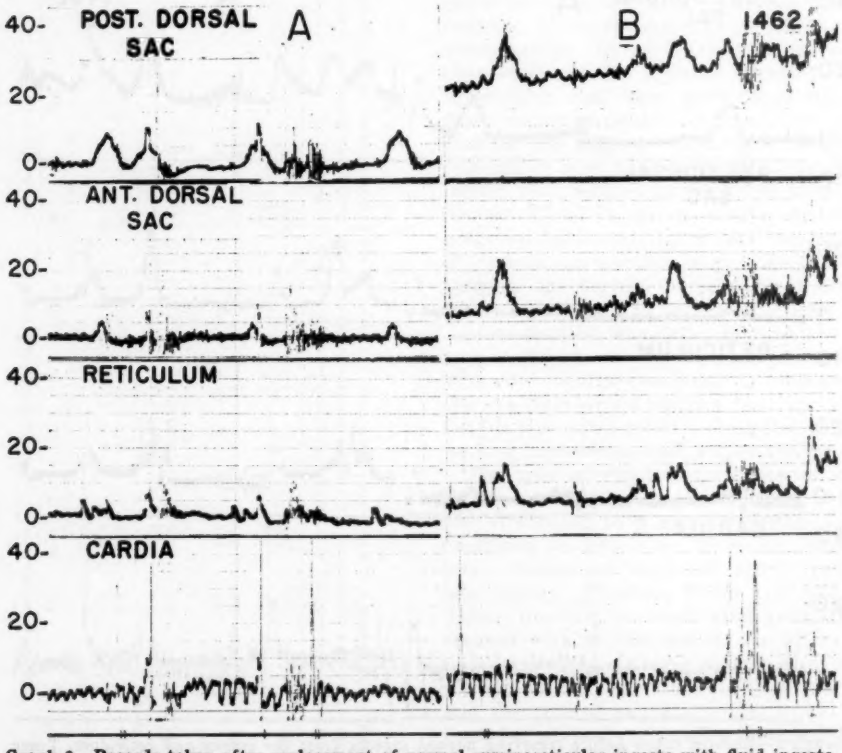
DISCUSSION

A study of the nervous reflexes associated with the rumen and reticulum must answer, among other things, the question of how many types of receptors are involved and whether two or more functions are mutually interdependent. For example, the rate of rumen contraction could be dependent on the amount of pressure or tension on the rumen wall, and eructation could be elicited only by contraction of the rumen muscle. However, the eructation rate would then be dependent, indirectly, upon intraruminal pressure. The same would be true of a function initiated through a tactile receptor, since roughage should provide the greatest stimulus during the contraction phase of the organ containing it. Therefore, the greater the rate of contraction, the more stimulation provided.

A second problem is that of trying to characterize receptors by the use of artificial stimuli. A test tube brush could stimulate tactile, pressure, or tension receptors, depending on the force with which it was applied to the mucosa, while pressure applied only to the inner wall of the rumen or reticulum would stimulate both pressure and tension receptors.

However, if the following results are considered with the above problems in mind, there appear to be repeated indications of certain relationships discussed below.

The results of this study indicated that the ruminoreticular cycle and the secondary rumen contractions continued at a basic rate, even when the reticulum and



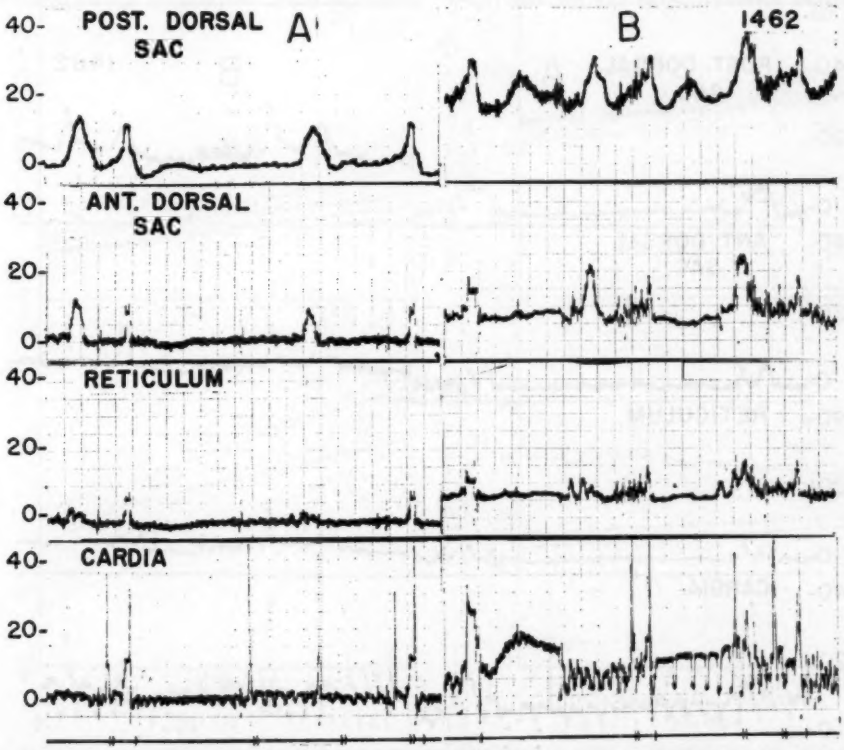
Graph 8—Records taken after replacement of normal ruminoreticular ingesta with fluid ingesta. Trace A was taken before and B during insufflation at 10 liters N_2/min. Notice decreased amplitude of rumen and reticular contractions and decreased number of eructations during insufflation, compared to graph 7 (normal ingesta) (animal 1462, 8-7-56).

rumen were entirely empty. Both functions showed an increase in rate with an increase in the pressure or tension on the forestomach walls. However, in addition, the rate of ruminoreticular contraction appeared to be influenced by other stimuli from this area. The results of the series of experiments on local stimulation of the ruminoreticular mucosa suggested that the rate of this cycle may also be under the influence of tactile stimulation.

Since stimulation of the ruminoreticular mucosa in the area of the cardia appeared to increase the rate of ruminoreticular contraction, this would suggest that an important number of receptors for this reflex was located here. Local stimulation or anesthetization of the rumen mucosa seemed to have little effect on the cycle.

Stimulation of either the ventral or dorsal abdominal vagus and stimulation of the cardiac mucosa increased the rate of secondary rumen contraction. However, this rate was also markedly increased by stimulation of the ruminal nerves or local pressure on the mucosa of the posterior rumen, and markedly decreased by procainization of ruminal nerves or the topical application of anesthetic to the dorsal rumen mucosa. This indicated that an important number of receptors for a reflex influencing the rate of rumen contraction was located in the posterior sacs of the rumen.

These results agreed with the findings of other workers that eructation occurs in conjuction with rumen contraction.[6, 12, 41, 42] It was further observed that any procedure which affected eructation rate almost in-



Graph 9—Records taken after removal of all ruminoreticular contents and placing of a fluid-filled plastic bag in the posterior ventral sac of the rumen. Trace A was taken before and B during insufflation at 10 liters N_2/min. Notice similarity between the amplitude of rumen contractions here and those with normal ingesta (graph 7). Reticular contractions less in amplitude than those with fluid ingesta present (graph 8). Notice also that eructations occur regularly during insufflation but only in conjunction with a posterior dorsal sac contraction, despite the fact that the cardia was exposed to the gas continuously (animal 1462, 8-7-56).

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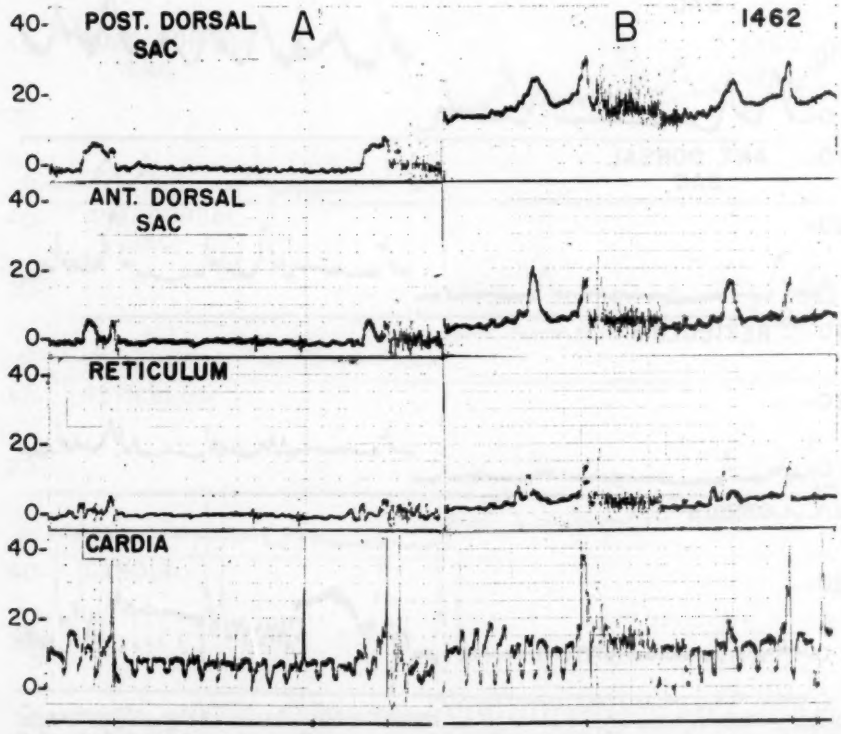
variably had a similar effect on the rate of rumen contraction. It appeared that the occurrence of eructation during rumen contraction was not simply a matter of the cardia being exposed to the gas at this time. Even with the reticulum empty (plastic bag series) or the entire ruminoreticular vault empty, eructation and rumen contraction were usually simultaneous. The fact that atropine inhibited eructation when the cardia remained exposed also suggests an association between muscle contraction and eructation. This question is quite critical to the understanding of the eructation reflex mechanism and requires further study.

Nerve stimulation and procainization further indicated that receptors initiating eructation were present in the posterior rumen and supported previous observations that afferent pathways for the reflex were located in the dorsal vagal trunk.[38]

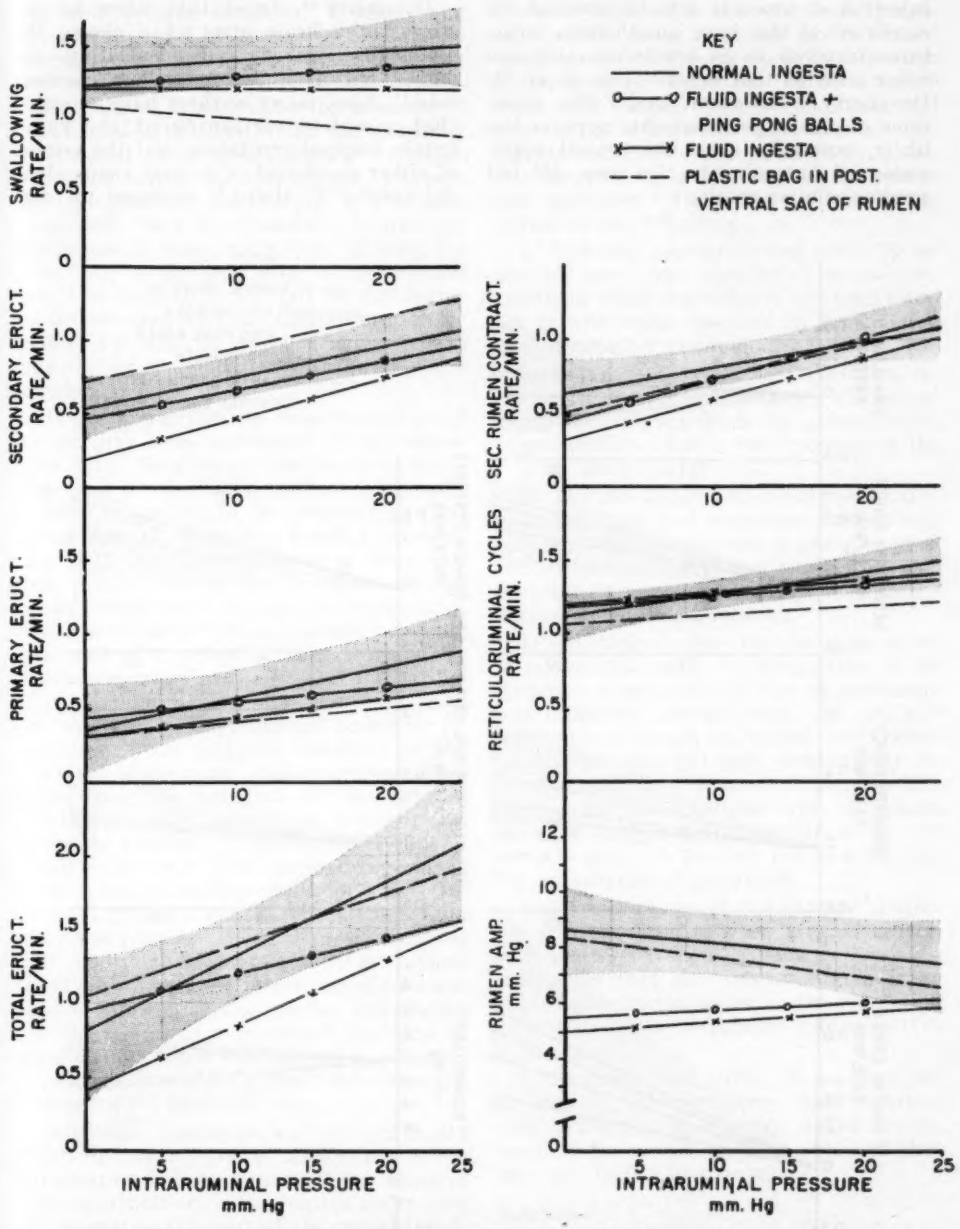
If eructation is initiated by rumen contraction but not by passive distention of the rumen, this would suggest two possible receptor mechanisms. The receptor could be a volume, or "in parallel" tension receptor in the rumen muscle, such as those demonstrated to be present in the heart and in the stomach of the cat. Alternatively, the receptor could be a tension receptor "in series" with the suspensory apparatus of the rumen.

The dissociation between eructation and rumen contraction after procainization of the cardia could be explained by the resulting relaxation of a normally constricted cardiac sphincter. This would allow gas to pass into the posterior esophagus. The intraesophageal pressure might then act to reflexly inhibit or mechanically overcome the other esophageal sphincters. The



Graph 10—Records taken after complete removal of ingesta from rumen and reticulum. Trace A was taken before and B during insufflation at 10 liters N_2/min. Notice amplitude of rumen and reticular contractions approach those seen with fluid ingesta on other days (graph 8). Also, eructation still accompanies the contraction of the posterior dorsal sac of the rumen (animal 1462, 11-23-57).

SWALLOWING
SECONDARY ERUCT.
PRIMARY ERUCT.
TOTAL ERUCT.



Graph 11—A series of graphs showing the effect of intraruminal pressure on eructation, swallowing, and ruminoreticular motility under 4 different conditions of ruminoreticular content. Each line represents the mean regression for 5 animals. The stippled areas represent the 95 per cent confidence interval determined for the regression lines of the normal ingesta series.

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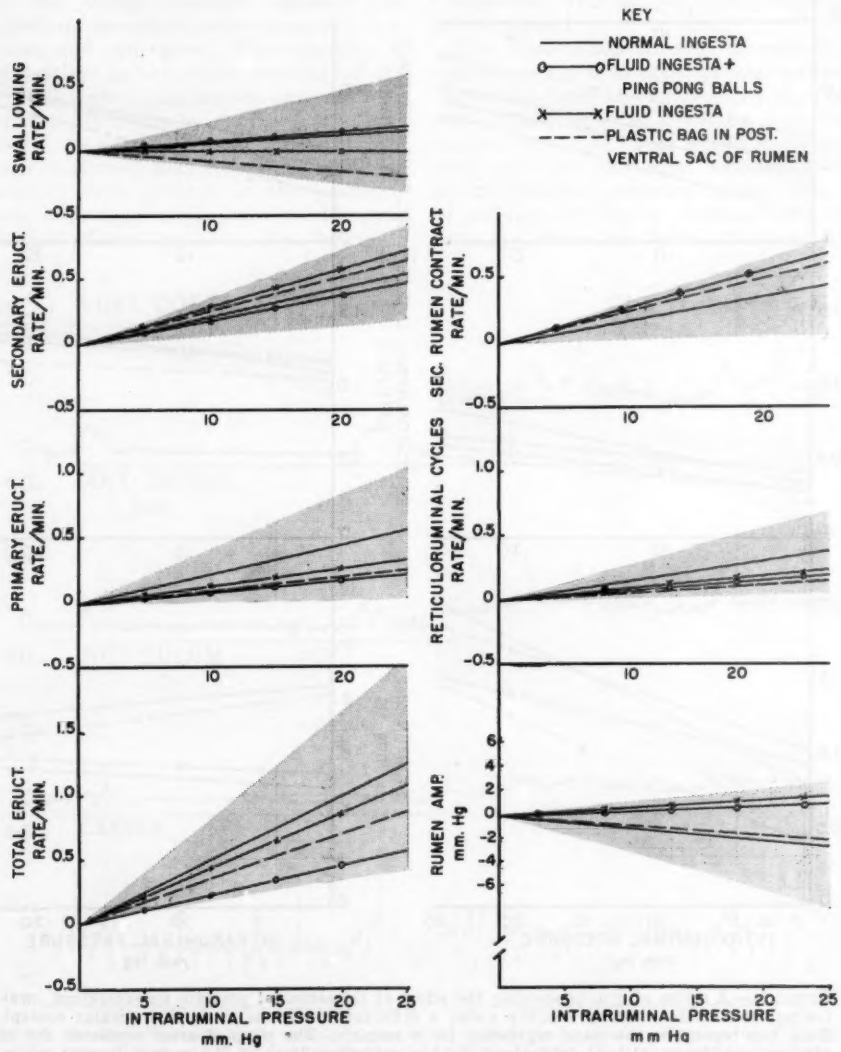
injection of procaine into the area of the cardia could also have anesthetized structures involved in an eructation-inhibition reflex such as that described in sheep by Dougherty.[11] However, under the conditions of this experiment this appears less likely, especially since the topical application of butacaine to this area did not produce similar results.

Dougherty[11] stated that when he destroyed the brain stem of a sheep, the esophagus relaxed and insufflating gas passed out at a "more or less constant rate." Also, many workers have reported that complete sectioning of the vagal trunks stopped eructation, but the section of either the dorsal or ventral vagus alone did not.[17,24,29] Habel[17] sectioned the dor-



Graph 12—A series of graphs derived from the same data as used in graph 11, but assuming the intercept equal to zero to allow comparison of slopes. The stippled areas represent the 95 per cent confidence interval for the slope of the regression lines of the normal ingesta series.

sal and ventral trunk of 2 sheep and found that, in 1 of these, "eructation occurred spontaneously and when the sheep was handled." Perhaps these various findings could be explained by the presence of esophageal sphincters which are normally constricted by a steady flow of impulses from the central nervous system. The acts of regurgitation, swallowing, and eructation could then be dependent, in part, on inhibition of these sphincters. If true, the effect of nerve sectioning on eructation could be dependent not only on the degree of interference with the afferent arm of the reflex, but also on whether motor nerves supplying the cardiac sphincter were severed.

It was hoped that the rate of swallowing might give some indication of the salivation rate. No attempt was made to establish such a correlation and certainly salivation is known to be dependent upon many factors other than a reflex originating in the ruminoreticular area. Nevertheless, it is of interest that the results of this study would tend to support the work of Clark and Weiss,[5] which demonstrated the presence of a salivation reflex elicited by tactile receptors in the area of the cardia. The ruminoreticular rate increased with an increase in intraruminal pressure, regardless of the physical character of the contents of the rumen and reticulum. However, this was not true of the rate of swallowing. An increase in intraruminal pressure resulted in an increased rate of swallowing only with normal ingesta or fluid plus ping-pong balls present. Increased pressure had no effect when fluid only was present. When the reticulum was left empty, there was actually an inverse relationship between intraruminal pressure and swallowing. Also, tactile stimulation of the cardia area increased the rate of swallowing, while the topical application of butacaine sulfate to this same area decreased the deglutition rate.

Although rumination was not specifically studied, the results of abdominal nerve stimulation indicated that afferent fibers of the regurgitation or rumination reflex may be present in the ventral abdominal trunk.

Summary

Some aspects of the reflex control of ruminoreticular motility and eructation were studied in adult, unanesthetized cows. Pressure changes in the reticulum, rumen, and cardia were recorded by means of strain gauge transducers activated through Brody-Quigley catheters. Measurements were made under normal conditions and then under a series of experimental conditions designed to either inhibit or stimulate the reflexes originating in the rumen and reticulum. The results of this study indicated the following:

1) Although eructation was normally associated with most secondary ruminal contractions, about one third of the total number of eructation occurred in conjunction with a primary ruminal contraction.

2) Afferent fibers for the eructation reflex were present in the dorsal abdominal vagus and afferent fibers for a rumination (regurgitation) reflex were present in the ventral abdominal vagus.

3) The rate and amplitude of the ruminoreticular cycle and secondary rumen contractions were dependent, in part, on pressure or tension. The rate of ruminoreticular contraction may also have depended on the degree of tactile stimulation.

4) Eructation most regularly occurred in conjunction with the contraction of the posterior rumen and the rate of eructation was found to correlate with the rate and amplitude of rumen contraction, even when the cardia was exposed continuously to intraruminal gas. This would suggest that the receptor is a volume type of tension receptor in the ruminal muscle, or an "in series" type of tension receptor in the rumen suspensory apparatus.

5) Procainization of the cardiac sphincter area appeared to allow a rapid eructation dissociated from rumen contraction, suggesting that this sphincter area is an important effector organ in the eructation reflex and one normally under active contraction.

6) Evidence was given to support the findings of other workers that receptors responsible for a salivation reflex are located in the cardia-reticular area and that they are tactile in nature.

References

[1] Ash, R. W., and Kay, R. N. B.: The Responses of the Reticulum and Parotid Gland of the Conscious Sheep to Stimulation of the Forestomachs. J. Physiol., *139*, (1957): 23.

[2] Aviado, D. M., Jr., and Schmidt, C. F.: Reflexes from Stretch Receptors in Blood Vessels, Heart and Lungs. Physiol. Rev., *35*, (1955): 247–300.

[3] Carpenter, F. W.: Nerve Endings of Sensory Type

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in the Muscular Coat of the Stomach and Small Intestine. J. Comp. Neurol., *29*, (1918): 553–560.

[4] Clark, C. H.: The Nerve Control of Rumination and Reticuloruminal Motility. Am. J. Vet. Res., *14*, (July, 1953): 376–384.

[5] Clark, R., and Weiss, K. E.: Reflex Salivation in Sheep and Goats Initiated by Mechanical Stimulation of the Cardiac Area of the Forestomachs. J. South African Vet. M. A., *23*, (1952): 163–165.

[6] Cole, H. H., Mead, S. W., and Kleiber, M.: Bloat in Cattle. Calif. Agric. Exp. Sta. Bull. 662, 1942.

[7] Comline, R. S., and Titchen, D. A.: Reflex Contractions of the Reticulum and Rumen and Parotid Salivary Secretion. J. Physiol., *139*, (1957): 24.

[8] Dawes, G. S.: Reflex Factors in the Regulation of the Circulation. Tr. Third Josiah Macy Jr. Found. Conf. Shock & Circulatory Homeostasis. H. D. Green, ed. Josiah Macy Jr. Foundation, New York, N.Y., 1954.

[9] Dougherty, R. W.: A Study of Drugs Affecting the Motility of the Bovine Rumen. Cornell Vet., *32*, (1942): 269–280.

[10] Dougherty, R. W., and Habel, R. E.: The Cranial-Esophageal Sphincter, Its Action and Its Relation to Eructation in Sheep as Determined by Cinefluorography. Cornell Vet., *45*, (1955): 459–464.

[11] Dougherty, R. W., Habel, R. E., and Bond, H. E.: Esophageal Innervation and the Eructation Reflex in Sheep. Am. J. Vet. Res., *19*, (Jan., 1958): 115–128.

[12] Dougherty, R. W., and Meridith, C. W.: Cinefluorographic Studies of the Ruminant Stomach and Eructation. Am. J. Vet. Res., *16*, (Jan., 1955): 96–100.

[13] Duncan, D. L.: The Effects of Vagotomy and Splanchnotomy on Gastric Motility in Sheep. J. Physiol., *119*, (1953): 157–169.

[14] Dussardier, M.: Controle Nerveux du Rythme Gastrique des Ruminants. J. physiol., Paris, *47*, (1955): 170–173.

[15] Dziuk, H. E., and Sellers, A. F.: Physiological Studies of the Vagal Nerve Supply to the Bovine Stomach. II. Studies on the Eructation Mechanism in Adult Cattle. Am. J. Vet. Res., *16*, (Oct., 1955): 499–504.

[16] Ellenberger, W.: Handbuch der Vergleichenden Mikroskopischen Anatomie der Haustiere. Vol. 3. Paul Parey, Berlin, 1911.

[17] Habel, R. E.: A Study of the Innervation of the Ruminant Stomach. Cornell Vet., *46*, (1956): 555–628.

[18] Habel, R. E., *op. cit.*, p. 575, citing Mangold, 1929: Handbuch der Ernährungund des Stoffwechsel der Landwirtschaftlichen Nutztiere als Grundlagen der Futterungslehre, Vol. 2, Springer, Berlin.

[19] Habel, R. E., *op. cit.*, p. 575, citing Marchall, 1910: Über den Einfluss des N. Vagus auf die Bewegungen des Magens der Wiederkäuer und über das Auftreten Einer Antral furche am Labmagen dieser Tiere. Diss., Bern, 1910.

[20] Henry, J. P., Gauer, O. H., and Reeves, J. L.: Evidence of the Atrial Location of Receptors Influencing Urine Flow. Circulation Res., *4*, (1956): 85–90.

[21] Henry, J. P., and Pierce, J. W.: The Possible Role of Cardiac Atrial Fibers in the Induction of Changes in Urinary Flow. J. Physiol., *131*, (1956): 572–580.

[22] Hill, C. J.: A Contribution to Our Knowledge of the Enteric Plexuses. Phil. Trans. Roy. Soc. London, *215*, (1927): 355–387.

[23] Hill, K. J.: Nervous Structures in the Reticuloruminal Epithelium of Goats. J. Physiol., *139*, (1957): 4.

[24] Hoflund, S.: Untersuchungen über Störungen in den Funktionen der Wiederkäuermagen durch Schadigungen des N. Vagus Verusacht. Svensk Vet.-tidskr., Suppl. to Vol. 45, 1940.

[25] Iggo, A.: Tension Receptors in the Stomach and the Urinary Bladder. J. Physiol., *128*, (1955): 593–607.

[26] Iggo, A.: Central Nervous Control of Gastric Movements in Sheep and Cattle. J. Physiol., *131*, (1956): 248–256.

[27] Iggo, A.: Gastric Mucosal Chemoreceptors with Vagal Afferent Fibers in the Cat. Quart. J. Exptl. Physiol., *42*, (1957): 398–409.

[28] Kay, R. N. B., and Phillipson, A. T.: Salivary Secretion of Anesthetized Sheep During Distension of the Rumen and Oesophagus. J. Physiol., *139*, (1957): 7.

[29] Mangold, E., and Klein, W.: Bewegungen und Innervation des Wiederkäuermagens. Georg Theime Verlag, Stuttgart, 1927.

[30] Mitchell, G. A. G.: Cardiovascular Innervation. E. & S. Livingstone Ltd., London, (1956): 90–111.

[31] Nettleship, W. A.: Experimental Studies on the Afferent Innervation of the Cat's Heart. J. Comp. Neurol., *64*, (1936): 115–131.

[32] Nichols, R. E.: A Note on Eructation in Sheep as Related to Rumen Fluid Level and Gas Insufflation. Am. J. Vet. Res., *12*, (July, 1951): 199–200.

[33] Nonidez, J. F.: Identification of Receptor Areas in the Venae Cavae and Pulmonary Veins Which Initiate Cardiac Acceleration. Am. J. Anat., *61*, (1937): 203–217.

[34] Paintal, A. S.: A Study of Right and Left Atrial Receptors. J. Physiol., *120*, (1953): 596–610.

[35] Paintal, A. S.: A Study of Gastric Stretch Receptors. Their Role in the Peripheral Mechanism of Satiation of Hunger and Thirst. J. Physiol., *126*, (1954): 255–270.

[36] Popow, N. A., Kudrjavcow, A. A., and Krasousky, W. K.: Zur Frage der Innervation und Pharmakologie des Magent der Wiederkäuer. Arch. Tierernähr u. Tierzucht, *9*, (1933): 243–252 [abstr. in Jahresbericht Veterinar–Medizin, *54*, (1934): 104–105].

[37] Schalk, A. F., and Amadon, R. S.: Physiology of the Ruminant Stomach (Bovine). North Dakota Agric. Exper. Sta. Bull. 216, 1928.

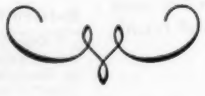
[38] Stevens, C. E., and Sellers, A. F.: Physiological Studies of the Vagal Nerve Supply to the Bovine Stomach. III. Procaine Effects on the Dorsal Vagal Trunk. Am. J. Vet. Res., *17*, (Oct., 1956): 588–598.

[39] Titchen, D. A. Reflex Contractions of the Reticulum. J. Physiol., *122*, (1953): 32.

[40] Von Grau, H., and Walter P.: Über die Feinere Innervation der Vormagen der Wiederkäuer. Acta anat., *31*, (1957): 21–35.

[41] Weiss, K. E.: Physiological Studies on Eructation in Ruminants. Onderstepoort J. Vet. Res., *26*, (1953): 251–283.

[42] Williams, E. J.: A Study of Reticuloruminal Motility in Adult Cattle in Relation to Bloat and Traumatic Gastritis. I. A Study of Reticuloruminal Motility in Adult Cattle with Reference to the Eructation of Ruminal Gases and Bloat. Vet. Med., *67*, (1955): 907–911.



The Pharmacological Action Developed in Cattle by Two Halogenated Sulfapyrimidines—Bromosulfamerazine and Bromosulfamethazine

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HALOID SULFONAMIDES have been investigated by a host of researchers for the last ten years.

In 1946, English *et al.*[2] carried out a research program, both *in vivo* and *in vitro*, on bromosulfadiazine, bromosulfamethazine, and bromosulfamerazine, and concluded that their action could be compared with that of sulfathiazole and sulfadiazine.

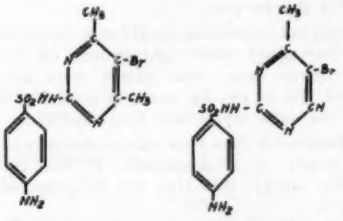
Waletsky and Hughes[9] reported that brominated, guanidinic sulfonamides were ten times as effective as the corresponding nonhalogenated compounds against avian coccidiosis.

Haloid sulfonamides are often found among chemicals tested on *Plasmodium gallinaceum* for their possible antimalarial action. In 1948, Little *et al.*[4] remarked that chloro- and bromosulfapyrimidine, as well as chloro- and bromopyrazine, together with the correspounding nonhalogenated compounds, had been tested against *Pasteurella multocida*. In 1950, Ishidate *et al.*,[5] among several other pyrimidinic and thiazolic derivatives considered, investigated the activity exerted *in vitro* on the *Mycobacterium tuberculosis* by five brominated thiazolic sulfonamides. Two of these proved active at a concentration of 50 μg. per milliliter. Beyer, as quoted by McManus *et al.*,[5] noticed that the halogenation of the number five carbon of the pyrimidine ring occasionally slowed down the elimination of these compounds through the urinary tracts of dogs.

In a research program on the effects of chloro- and bromosulfadiazine, chloro- and bromosulfamethazine, chloro- and bromosulfamerazine, and their nonhalogenated compounds on cattle, McManus *et al.*[5] in 1951 stressed the practical veterinary value of haloid sulfonamides as long-acting therapeutic agents. Sulfamerazine appeared to draw the greatest benefit from halogenation. However, the toxic properties of the drug were markedly increased, although not to the extent of sulfadiazine.

Bromosulfamethazine was studied by Stowe *et al.*[8] The satisfactory results they obtained by oral administration are comparable, in view of their preceding findings, to those that can be obtained from sulfamethazine or sulfamethoxypyridazine. They suggested, although with some reservation, that this compound could be profitably used in clinical practice.

The dosage adopted by Stowe *et al.*[8] was higher than we normally use, and we failed to see the advisability of increasing it for these sulfonamides, which are notoriously more toxic than those in common usage. Therefore, we proposed to investigate whether the advantages remain although conditions were different. Findings in respect to acute toxicity, activity *in vitro*, plasma concentrations after various time intervals, and binding with plasma proteins, as established for bromosulfamethazine and bromosulfamerazine, were compared with those for sulfamethazine and sulfamerazine, respectively.



2-sulfanilamido-4.6-dimethyl-5-bromopyrimidine. Bromosulfamethazine

2-sulfanilamido-4-methyl-5-bromopyrimidine. Bromosulfamerazine

Fig. 1—Bromosulfamethazine and bromosulfamerazine structures.

MATERIALS AND METHODS

All sulfonamide determinations (free, *i.e.*, nonacetylated sulfonamide, then the total sulfonamide after acid hydrolysis) were made with the colorimetric method of Bratton and Marshall.[1] The data were obtained by comparing the color intensity of unknown plasma samples with standard solutions prepared at concentrations of 0.5, 1.0, 3.0, 5.0, 7.0,

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Part of the investigation concerning acuted toxicity and activity *in vitro* was made possible through the cooperation of Dr. C. Bertazzoli and Dr. M. Ghione, Sezione di Microbiologia e di Chemioterapia, Farmitalia, Milan.

TABLE 1—Acute Toxicity Data Obtained from Intravenous Administration of Drugs to Mice

Drug	i.v. acute l.d.50 (mg./kg.)
Sulfamethazine	751
Bromosulfamethazine	529
Sulfamerazine	1,256
Bromosulfamerazine	668

9.0, 11.0, and 13.0 mg. per 100 ml. of plasma. A colorimeter* (filter No. 520) was used to measure optical density. Standard solutions for plasma were prepared by adding the required amounts of each drug to heparinized plasma obtained from the same animals prior to drug administration.

The acute toxicity data were obtained by injecting albino mice intravenously with the sodium salts of the drugs under consideration. Minimum inhibitive dosage (m.i.d.) was determined on a variety of cultures, with and without the addition of 10 per cent blood serum. The behaviour of brominated sulfonamides in respect to plasma proteins was observed *in vitro* by determining the percentage of drugs that could not be diffused through a cellophane membrane separating a sample of bovine plasma from a 0.15 M sodium chloride solution in a 0.01 M phosphate buffer (pH 7.4) containing the sulfonamides in various known concentrations. As the balance between the solutions was reached, the percentage of drug bound to the plasma proteins was determined by the difference between the total and the unbound drug. The colorimetric method of Bratton and Marshall was used for this purpose.

Plasma concentrations at different time intervals were determined with eight groups of 8 Brown Swiss calves each. The calves were all male, weighed 170 to 180 kg. each, in good health, and under the same stable and feed conditions.

Sulfonamides were each administered in a single dose, orally or intravenously, in the following amounts: orally, 0.15 Gm. per kilogram of body weight; intravenously, 0.075 Gm. per kilogram of body weight.

Oral drugs were given in boluses of honey and licorice powder. Drugs administered intravenously were in the form of sodium salts in aqueous solutions with the following concentrations: sulfamethazine and sulfamerazine, 20 per cent; bromosulfamethazine, 10 per cent; and bromosulfamerazine, 5 per cent. The solutions of the two halogenated sulfonamides were prepared in a considerably lower range of concentration because of their lower solubility.

* Hellige CliniCol Photoelectric Colorimeter, manufactured by Hellige, Inc., Garden City, N.Y.

RESULTS

Halogenated sulfonamides were found to have a higher toxicity than the corresponding nonhalogenated structures (table 1). Bromosulfamerazine appeared to be approximately twice as toxic as nonhalogenated sulfamerazine.

The *in vitro* activity of halogenated sulfonamides was generally either equal to or higher than that of nonhalogenated ones, and in many cases the activity of the former was impaired to a lesser extent by the presence of blood serum (table 2).

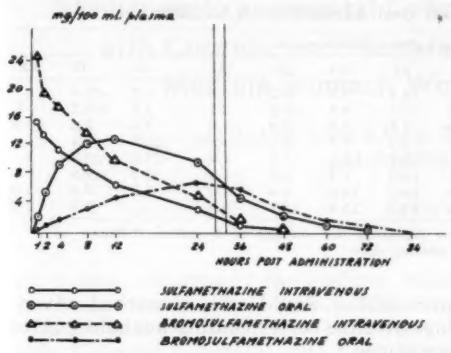
In agreement with the findings of McManus *et al.*,[5] brominated sulfonamides were bound to plasma proteins in percentages considerably higher than those of nonbrominated sulfonamides (table 3). These percentages for low concentrations exceed 90 per cent.

From the plasma concentration values after intravenous administration (table 4), it appeared that considerably higher values were maintained by the halogenated derivatives than by the nonhalogenated ones during the first few hours. The values for bromosulfamethazine, 24 hours after administration, were nearly identical to those observed for sulfamethazine (graph 1).

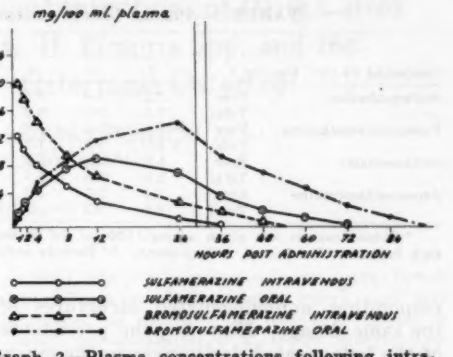
TABLE 2—Minimum Inhibitive Dosages

		m.i.d. μg./ml.							
		Sulfamethazine		Bromosulfamethazine		Sulfamerazine		Bromosulfamerazine	
Medium	Organism	No serum added	10% blood serum added	No serum added	10% blood serum added	No serum added	10% blood serum added	No serum added	10% blood serum added
Broth—yeast	*B. subtilis*	20	100	20	50	20	100	10	25
Medium for *L. casei*	*B. subtilis*	10	10	5	5	50	50	5	5
Medium for *L. casei*	*S. faecalis*	50	>50	50	>50	50	>50	50	>50
Medium for *L. casei*	*N. asteroides*	10(5)	50	2.5	10	10	50	5(2)	10
Medium for *L. casei*	Staph. 114	5(2)	50	5(2)	50	10(5)	50	5(2)	10
Medium for *L. casei*	*S. agalactiae*	50	100	10	50	10	100	10	50
Medium for *L. casei*	*S. dysgalactiae*	10	100	5	50	5	100	5	50
Medium for *L. casei*	*S. uberis*	50	100	50	50	50	100	50	50
Medium for *L. casei*	*Esch. coli*	50	>50	50	>50	50	>50	50	>50
Sayum's medium	*Esch. coli*	10	10	50	100	10	10	10	10

Figures in parentheses represent the semi-inhibition dose.



Graph 1—Plasma concentrations following intravenous and oral administration of sulfamethazine and bromosulfamethazine to calves.



Graph 2—Plasma concentrations following intravenous and oral administration of sulfamerazine and bromosulfamerazine to calves.

However, although the sulfamerazine contents were already hardly detectable after 24 hours, traces of bromosulfamerazine were still noticeable after as many as 60 hours (graph 2).

When administered orally (table 5, graph 1), bromosulfamethazine appeared to be absorbed quite slowly; its peak plasma concentration was not reached until 24 hours after administration, when its concentration value was only slightly higher than that achieved by nonhalogenated sulfamethazine after just two hours. These data, which were not reported by McManus *et al.*,[5] appear to be inconsistent with their suggestion that absorption of sulfonamides would be assisted by halogenation. On the contrary, although bromosulfamerazine closely followed the pattern of the corresponding nonhalogenated compound for the first 8 hours, its concentration values appeared to be considerably higher thereafter, and its presence, though only in limited amounts, was detectable up to 84 hours after administration (table 5, graph 2).

DISCUSSION

The preceding findings seem to warrant the assumption that the intravenous administration of halogenated derivatives would make it possible to reach higher plasma concentrations than with the cor-

TABLE 3—Plasma Protein Binding

Drug	Total drug (mg./100 ml. of plasma)	Bound drug (%)
Sulfamethazine	5.5	67
	8.6	66
	12.0	61
Bromosulfamethazine	3.2	95
	7.0	90
	12.0	86
Sulfamerazine	4.0	52
	5.0	40
Bromosulfamerazine	5.5	92
	7.0	89
	11.0	83

TABLE 4—Plasma Concentrations in Calves After Intravenous Administration

Compound (0.075 Gm./kg.)		Hours after administration								
		1	2	4	8	12	24	36	48	60
Sulfamethazine	Free	15.4*	13.6	11.5	8.5	6.5	3.1	1.0	+†	0
	Total	16.3	14.7	12.1	9.1	7.2	3.4	1.2	+	<0.5
Bromosulfamethazine	Free	24.4	19.4	17.5	13.7	10.0	5.1	1.0	<0.5	+
	Total	25.1	22.6	18.8	15.3	.....**	....	....	....	+
Sulfamerazine	Free	12.6	10.3	7.8	5.3	3.1	<1.0	+	0	0
	Total	13.4	.....	.....	.....	3.6	1.1	+	0	0
Bromosulfamerazine	Free	20.0	16.8	14.0	9.0	6.3	3.0	1.6	1.1	0
	Total	24.4	19.5	15.3	10.5	6.8	3.8	....	....	0

* Concentrations are given as mg./100 ml. of plasma and represent the average of 8 calves. † Plus sign indicates that traces were present. ** Periods indicate missing data.

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TABLE 5—Plasma Concentrations After Oral Administration to Calves

Compound (0.150 Gm./kg.)		Hours after administration										
		1	2	4	8	12	24	36	48	60	72	84
Sulfamethazine	Free	2.2*	5.4	9.1	12.1	12.3	9.3	5.2	2.5	1.0	<0.7	0
	Total	2.5	6.0	9.3	13.2	13.3	9.8	6.2	2.9	1.3	<0.7	+†
Bromosulfamethazine	Free	<0.7	<1.0	1.3	3.0	4.7	6.6	5.4	3.4	1.5	1.4	<1.0
	Total	<1.0	1.0	1.3	3.6	5.9	7.4	6.1	....**	<2.8	1.4	<1.0
Sulfamerazine	Free	1.5	3.0	5.2	8.1	9.3	7.4	3.6	1.6	<1.0	<0.5	0
	Total	2.0	3.5	5.7	8.7	10.0	7.4	3.9	1.9	1.1	<0.5	0
Bromosulfamerazine	Free	1.4	2.5	5.7	8.6	12.0	14.5	9.4	7.3	5.2	2.6	1.4
	Total	1.9	3.3	....	10.2	13.2	15.9	11.7	8.6	....	2.9	1.6

* Concentrations are given as mg./100 ml. of plasma and represent the average of 8 calves. † Plus sign indicates that traces were present. ** Periods indicate missing data.

responding nonhalogenated structures of the same dosage. However, the persistence of the halogenated derivatives in the circulation would not be prolonged considerably.

As to oral administration, while poor results are obtained with bromosulfamethazine, bromosulfamerazine appears to remain at a probably therapeutic concentration level for nearly 60 hours after a single-dose administration.

In view of the toxic properties of these compounds and of their questionable higher bacteriostatic activity, it is felt that no conclusion should be ventured about a definite therapeutic superiority of brominated derivatives over the corresponding nonhalogenated structures until the results of further clinical tests will be published to integrate those that are being made by researchers as quoted in personal communications to Stowe *et al.*[8]

SUMMARY

The pharmacological behaviour of two halogenated sulfapyrimidines (bromosulfamerazine and bromosulfamethazine) in cattle was compared to that of the corresponding nonhalogenated structures with a view of an eventual therapeutic use.

There seemed to be no grounds for supporting the assumption that bromosulfapyrimidines would offer a marked advantage over the corresponding nonhalogenated structures.

References

[1] Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determinations. J. Biol. Chem., *128*, (1939): 537–550.

[2] English, J. P., Clark, T. H., Maison, H. W., Clapp, J. W., Seeger, D., and Ebel, R. H.: Studies in Chemiotherapy. XII. Antimalarials. J. Am. Chem. Soc., *68*, (1946): 453–458.

[3] Ishidate, Morizo, Miura, Yoshiaki, and Yamamoto, Yuichi: Chemiotherapy of Tuberculosis. II. *In Vitro* Activity of Pyridine and Thiazole Derivatives. Abstr. in Chem. Abstr., *48*, (1954): 7113.

[4] Little, P. A., De Mello, G., Tanzola, F., and Subbarow, Y.: Comparison of Mouse-Protective Test and Chick-Protective Test of Over 600 Chemicals Against *Pasteurella Multocida*. J. Immunol., *60*, (1948): 295–298.

[5] McManus, E. C., Scheidy, S. F., Angstadt, B. S., McKinney, S. E., Pitt, A. A., and Schuchardt, G. S.: Experimental Evaluation of Six Halogenated Sulfapyrimidines in Cattle. Am. J. Vet. Res., *12*, (April, 1951): 81–84.

[6] Roy, A. C., Guha, S. S., Keshavamurthy, N. K., Chandrasekhar, G. R., Menon, K. P., and Guha, P. C.: Chemiotherapy of Malaria. Abstr. in Chem. Abstr., *48*, (1954): 4116.

[7] Schneider, J., and Decourt, P.: Avian Malaria and Chemiotherapy. Proc. 11th Internat. Cong. Pure Applied Chem. (London), *3* (1947): 339 [abstr. in Chem. Abstr., *46*, (1952): 7657.]

[8] Stowe, C. M., Aronson, A. L., and Johnson, K.: The Pharmacology of Sulfabromomethazine, a New Long-Acting Sulfonamide, in Cattle. Am. J. Vet. Res., *19*, (April, 1958): 345–353.

[9] Waletsky, E., and Hughes, C. O.: The Relative Activity of Sulfonamides and Other Compounds in Avian Coccidiosis (*Eimeria Tenella*). Am. J. Vet. Res., *7*, (July, 1946): 365–373.

Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. II. Eimeria Spp. and the Medium Stomach Worm, *Ostertagia Ostertagi*

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IN A PREVIOUS STUDY,[6] the authors found that experimental concurrent infections with Eimeria spp. and an intestinal nematode, *Cooperia punctata*, were more injurious to calves than infection with either one alone. Consequently, it was decided to conduct similar tests, substituting the medium stomach worm, *Ostertagia ostertagi*, for the intestinal worm used previously. The object was to determine whether these two parasites which localize in different portions of the alimentary tract would, when inoculated at the same time, (1) be more injurious to the host than either one alone, and (2) affect the development of each other. The investigation was performed during the period of September to December, 1954.

MATERIALS AND METHODS

Male calves, 5 to 31 days old at the beginning, were utilized as experimental animals for two tests. They were either grade Jerseys or Jersey-Guernsey crosses, born on farms in the vicinity of Auburn, Ala., and purchased when 1 to 2 days old. They had been exposed to natural infections of coccidia at birth or shortly thereafter. Additional infections probably were acquired between the time they were moved to the laboratory and the start of the experiments. The usual result of exposure of calves to natural light infections of coccidia was described in a previous report.[3]

Prior to initiation of each test, the calves involved were maintained under conditions similar to those previously described.[6] Subsequent to initiation of the experiment, the animals were housed in duplex concrete-floored pens constructed of hollow tile and concrete.[5] Each day the calves were moved to the cleaned side of their double unit.

In test 1, the 12 calves were allotted to four groups of 3 each. In test 2, the 8 calves remaining from test 1 were assigned to four groups of 2 calves each. In each test, one group was experimentally infected with coccidia (Eimeria) only. Another group was given coccidia plus infective larvae of the medium stomach worm, *O. ostertagi*. A third group was given stomach worm larvae only. Calves in the remaining group were left uninfected as controls. In test 1, 1 of the 3 control calves developed a severe diarrhea on the fourth day and was eliminated from the experiment.

Eimeria Infection.—Coccidial oocysts used in the investigation were obtained from naturally or experimentally infected calves. The species and percentages of each were as follows: *Eimeria alabamensis*, 30; *Eimeria zurnii*, 27 (spherical forms, 19; elliptical forms, 8); *Eimeria ellipsoidalis*, 18; *Eimeria cylindrica*, 8; *Eimeria subspherica*, 8; *Eimeria bovis*, 7; and *Eimeria auburnensis*, 2.

In the first test, calves given coccidia only were each fed 2,000 sporulated oocysts per pound of body weight on experimental days 1, 3, and 4, a total of 6,000 oocysts per pound. These oocysts, contained in a moist paste, were applied to the base of the tongue by a wooden tongue depressor.

Ostertagia Infection.—Stomach worm (Ostertagia) larvae were cultured from the feces of animals harboring pure infections. In test 1, the 3 calves inoculated with stomach worm larvae only were each given a total of 75,000 larvae in three equal doses on experimental days 1, 3, and 4. Each of the calves inoculated with Eimeria and Ostertagia was given one half the number of larvae and coccidia given to the calves inoculated with only one of the two parasites. In the first test, the larvae were administered, at the same time as the oocysts in three equal doses by a metal pipette.

In test 1, 1 calf from each of the three infected groups was killed for necropsy 21 days after the first infection to determine (1) whether damage had resulted to the digestive tract, (2) the nature and extent of this damage, and (3) the extent to which the worms and coccidia had developed during the 21-day period. The surviving 8 calves were weaned after 37 days of the experiment and used in a second test.

In test 2, the calves that had been inoculated with Eimeria spp. in test 1 were given twice as many oocysts per pound because of the increased ages of the experimental animals. The oocysts were from the same culture used in test 1 and were given in three inoculations at the same time intervals as before.

The calves that had been given larvae before were given slightly more than twice as many in the second test (160,000 and 80,000 per calf to the groups given Ostertagia and to those given the Ei-

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TABLE 1—A Comparison of Calves Exposed to Eimeria spp., Ostertagia Ostertagi, or a Combination of Both, and Controls (Test 1)

Group and calf No.	Age (days)	No. times signs of infections were evident in fecal samples			Oocysts				Weights gained or lost (lb.) by weeks, since initial weight						
		Mucus	Blood	Diarrhea	0–100*	100–500	500–1,000	1,000–10,000	10,000+	Initial weight	1	2	3	4	5	6	7
EIMERIA GROUP																	
1382	7.0	0.0(0.0)†	0.0(0.0)	3.0(6.0)	6.0(2.0)	0.0(3.0)	0.0(1.0)	9.0(6.0)	0.0(0.0)	60.0	2.0	1.0	3.0	4.0	7.0	16.0	20.0
1371	23.0	1.0(0.0)	1.0(0.0)	3.0(0.0)	7.0(6.0)	1.0(1.0)	2.0(0.0)	5.0(5.0)	0.0(0.0)	58.0	5.0	10.0	13.0	21.0	29.0	38.0	39.0
1386	5.0	0.0 (....)	1.0 (....)	8.0 (....)	7.0 (....)	3.0 (....)	1.0 (....)	4.0 (....)	0.0 (....)	63.0	−9.0	−6.0	−8.0	Killed on day 21			
Av.	11.7	0.3(0.0)	0.7(0.0)	4.7(3.0)	6.7(4.0)	1.3(2.0)	1.0(0.5)	6.0(5.5)	0.0(0.0)	60.3	−0.6	1.7	2.7	12.5	18.0	27.0	29.5
OSTERTAGIA GROUP																	
1379	13.0	0.0(0.0)	0.0(0.0)	0.0(4.0)	13.0(4.0)	2.0(3.0)	0.0(4.0)	0.0(1.0)	0.0(0.0)	49.0	−1.0	1.0	3.0	8.0	14.0	23.0	16.0
1373	20.0	0.0(0.0)	0.0(0.0)	0.0(0.0)	10.0(9.0)	2.0(3.0)	2.0(0.0)	0.0(0.0)	0.0(0.0)	59.0	2.0	3.0	7.0	17.0	25.0	35.0	24.0
1387	5.0	0.0 (....)	0.0 (....)	2.0 (....)	11.0 (....)	3.0 (....)	0.0 (....)	0.0 (....)	0.0 (....)	64.0	−7.0	−9.0	−5.0	Killed on day 21			
Av.	12.7	0.0(0.0)	0.0(0.0)	0.7(2.0)	11.3(6.5)	2.3(3.0)	0.7(2.0)	0.0(0.5)	0.0(0.0)	57.3	−2.0	−1.7	1.7	12.5	19.5	29.0	20.0
EIMERIA-OSTERTAGIA GROUP *(one half the numbers of each)*																	
1383	6.0	0.0(0.0)	0.0(0.0)	2.0(3.0)	7.0(3.0)	1.0(0.0)	0.0(0.0)	5.0(8.0)	2.0(1.0)	54.0	−1.0	−4.0	3.0	11.0	25.0	19.0	19.0
1370	23.0	2.0(0.0)	1.0(0.0)	0.0(1.0)	4.0(3.0)	0.0(3.0)	0.0(2.0)	11.0(4.0)	0.0(0.0)	61.0	1.0	−4.0	11.0	15.0	8.0	26.0	32.0
1385	5.0	0.0 (....)	0.0 (....)	2.0 (....)	11.0 (....)	0.0 (....)	0.0 (....)	4.0 (....)	0.0 (....)	58.0	−4.0	−4.0	3.0	Killed on day 21			
Av.	11.3	0.7(0.0)	0.3(0.0)	1.3(2.0)	7.3(3.0)	0.3(1.5)	0.0(1.1)	6.7(6.0)	0.7(0.5)	57.7	−1.3	−1.3	5.7	13.0	16.5	22.5	25.5
CONTROLS																	
1367	31.0	1.0(0.0)	0.0(0.0)	0.0(0.0)	13.0(4.0)	2.0(3.0)	0.0(2.0)	0.0(3.0)	0.0(0.0)	57.0	4.0	4.0	15.0	21.0	28.0	38.0	38.0
1377	16.0	1.0(1.0)	0.0(0.0)	2.0(1.0)	9.0(12.0)	3.0(0.0)	1.0(0.0)	2.0(0.0)	0.0(0.0)	64.0	−5.0	−3.0	0.0	8.0	11.0	18.0	20.0
Av.	23.5	1.0(0.5)	0.0(0.0)	1.0(0.5)	11.0(8.0)	2.5(1.5)	0.5(1.0)	1.0(1.5)	0.0(0.0)	60.5	−0.5	0.5	7.5	14.5	19.5	28.0	29.0

* Oocyst count per loopful.[1] † Each unenclosed number represents 15 daily fecal samples (except for 1373 and 1387, with 14 each) obtained from the fourth to the twenty-first day after the first inoculation. Each number in parentheses represents 12 daily samples obtained during the ensuing two weeks.

meria and Ostertagia, respectively). These larvae, given in a single dose on the first day of the second experiment, had been cultured from the feces of the calves infected during the first experiment and from another experimentally infected calf.

Blood samples were taken from all calves weekly, and packed cell volumes were determined by centrifuging 1 ml. of oxalated blood in Wintrobe hematocrit tubes at 3,600 r.p.m. for one hour.

Necropsies of calves that were killed or that died during the two tests included studies of fresh intestinal smears prepared in saline solution and of stained sections of tissues fixed in Zenker's or Technicon fixatives. The worms were recovered and the numbers determined by the technique described by Porter.[9]

RESULTS AND DISCUSSION

Test 1.—In test 1, (table 1) there was little difference in maximum oocyst counts in the two groups given coccidia, but 1 calf (1383) with mixed infections did show oocyst counts of over 10,000 per loopful [1] on experimental days 20, 21, and 22. These peaks were predominantly *E. ellipsoidalis*, with *E. zurnii* and *E. cylindrica* included.

Examination of feces by the direct centrifugal flotation technique showed 2 of the 3 calves infected with worms only and 1 of the 3 with both infections were passing fewer than 10 eggs per gram of feces (e.p.g.) 31 days after the inoculations.

During the first 21 days, the calves inoculated with Eimeria spp. only, had diarrhea about twice as often, on the average, as calves in the other two parasitized groups combined (table 1). The differences in weights in all four groups throughout the test were inconsequential.

No significant changes occurred in packed blood cell volumes in any of the calves.

Necropsy Findings, Test 1.—When killed for necropsy 21 days after inoculations, the findings were as follows:

Calf 1386 (Eimeria).—The small intestine, particularly the posterior third, and the entire colon were hyperemic. The mesenteries were not edematous, however. Merozoites of coccidia were observed in small numbers in the anterior 10 feet of the small intestine, were not seen in the next 12 feet, then reappeared 30 feet anterior to the ileocecal valve in increasing numbers to a point 1 foot from the valve. None was found in either the cecum or the colon. Oocysts of *E. ellipsoidalis* were found 24 feet anterior to the ileocecal valve and also in the lower colon. Oocysts of *E.*

zurnii were seen in the lower colon. There were small numbers of trichomonads in the cecum, colon, and rectum and numerous Giardia in the middle region of the small intestine. The latter were most numerous (28 per high-dry objective field) in an area about 12 feet anterior to the ileocecal valve.

Calf 1387 (Ostertagia).—The abomasum was markedly edematous, and the mucosal surface contained many nodules typical of this species. Of the 680 worms recovered, representing less than 1 per cent of the infective larvae administered, 250 were larvae recovered from a portion of the abomasum macerated in a Waring blendor. Utilization of the pepsin-hydrochloric acid digestion technique, later developed by Herlich,[8] might have revealed additional worms.

Calf 1385 (Eimeria-Ostertagia).—There were swollen, reddened, longitudinal ridges about 3 inches long in the anterior 6 inches of the colon. These made the wall about twice as thick as in the other regions of the colon. Numerous merozoites were found throughout the small intestine, except in the anterior 10 feet. They were present in small numbers in the upper colon, but were not found in the remainder of the colon, in the cecum, or in the rectum. Small schizonts resembling those of *E. zurnii*[4] were seen 36 feet anterior to the ileocecal valve, and a few macroscopic schizonts similar to those of *E. bovis* were seen in the terminal 6 feet of the small intestine. Numerous oocysts of *E. zurnii* were located 30 and 24 feet above the ileocecal valve. Oocysts of *E. ellipsoidalis* were seen in the lower cecum, upper colon, and rectum. In the cecum and upper colon, there were a few trichomonads. Giardia, in small numbers, were seen in the lower cecum.

The abomasum of this animal was edematous, and nodules were present, although not so extensively as in calf 1387. The 419 worms recovered represented about 1 per cent of the infecting dose. Again, a small number of immature worms was recovered after a portion of the abomasum was macerated in a Waring blendor and more worms undoubtedly would have been recovered had the abomasum been subjected to digestion in pepsin and hydrochloric acid.

Test 2.—None of the calves in the four groups in test 2 (table 2) passed mucus or blood in the feces. Diarrhea, observed only in the calves in the groups infected with Eimeria and Eimeria-Ostertagia, occurred on about the same number of days in both groups. Fairly large numbers of oocysts were discharged by calves in both coccidia-infected groups. In only 1 calf (1382, Eimeria) was the peak of oocyst production greater than 10,000 per loopful.[1] In both Eimeria and Eimeria-Ostertagia infected groups, the dominant species in the daily peaks of 1,000 to 10,000 oocysts was *E. alabamensis*, which has been shown capable of reinfecting the same calf as many as four times.[2] *Eimeria subspherica* appeared as a natural infection in both calves in the Ostertagia group, and this species accounted for the numbers of oocysts listed for this group (table 2).

Significant changes occurred in packed blood cell volumes only in calf 1382, in which scouring and dehydration were apparently responsible.

Just prior to the beginning of test 2, the 2 calves with worm infection only showed fecal egg counts of 378 and 3,600 e.p.g., whereas the calves infected with both coccidia and worms had counts of 432 and 732 e.p.g. Worms were being eliminated with the feces of all 4 calves. These egg counts indicate a large number of worms; thus the extremely small numbers recovered from the calves killed in the first phase of the experiment probably resulted from a failure to recover worms still imbedded in the mucosa. The milk diet was probably not responsible. Subsequent investigations have shown that milk has no apparent effect on *O. ostertagi* infections.[10]

Despite the absence of mucus and bloody feces, the Eimeria and the Eimeria-Ostertagia infected calves had lost an average of 1.5 and 0.5 lb., respectively, by the third week of the second test, while the Ostertagia-infected and the control groups had gained an average of 19.0 and 9.0 lb., respectively. The younger calves in the three parasitized groups died on experimental days 26, 30, and 39, respectively. The fact that the younger calves in the Eimeria and the Eimeria-Ostertagia groups lost 9.0 and 7.0 lb., respectively, in three weeks and eventually died may indicate that, even though they had been inoculated in the previous experiment with oocysts from the same culture, they probably had not established as much resistance as the older calves.

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TABLE 2—A Comparison of Calves Exposed to Eimeria spp., Ostertagia Ostertagi, or a Combination of Both, and Controls (Test 2)

Group and calf No.	Age (days)	No. times signs of infections were evident in fecal samples			Oocysts				Weight gained or lost (lb.) by weeks, since initial weight					
		Mucus	Blood	Diarrhea	0–100*	100–500	500–1,000	1,000–10,000	10,000+	Initial weight	1	2	3	4	5	6
EIMERIA GROUP																
1382	44.0	0.0†	0.0	4.0	10.0	2.0	2.0	4.0	1.0	80.0	2.0	1.0	−9.0	Died, day 26		
1371	60.0	0.0	0.0	7.0	10.0	6.0	0.0	3.0	0.0	97.0	8.0	10.0	−6.0	15.0	19.0	13.0
Av.	52.0	0.0	0.0	5.5	10.0	4.0	1.0	3.5	0.5	88.5	5.0	5.5	−1.5	.....	.....	.....
OSTERTAGIA GROUP																
1379	50.0	0.0	0.0	0.0	10.0	2.0	2.0	5.0	0.0	65.0	−5.0	5.0	11.0	5.0	Died, day 30	
1373	57.0	0.0	0.0	0.0	16.0	1.0	1.0	1.0	0.0	82.0	20.0	20.0	27.0	30.0	36.0	32.0
Av.	53.5	0.0	0.0	0.0	13.0	1.5	1.5	3.0	0.0	73.5	7.5	12.5	19.0	17.5	.....	.....
EIMERIA-OSTERTAGIA GROUP (*one half the numbers of each*)																
1383	43.0	0.0	0.0	5.0	11.0	6.0	0.0	2.0	0.0	73.0	4.0	9.0	−7.0	11.0	11.0	5.0
1370	60.0	0.0	0.0	7.0	10.0	6.0	0.0	3.0	0.0	97.0	8.0	10.0	−6.0	15.0	19.0	13.0
Av.	51.5	0.0	0.0	6.0	10.5	6.0	0.0	2.5	0.0	85.0	6.0	9.5	−0.5	13.0	15.0	9.0
CONTROLS																
1367	66.0	0.0	0.0	0.0	17.0	2.0	0.0	0.0	0.0	95.0	5.0	11.0	8.0	13.0	23.0	21.0
1377	53.0	0.0	0.0	0.0	19.0	0.0	0.0	0.0	0.0	84.0	3.0	16.0	10.0	10.0	17.0	17.0
Av.	60.5	0.0	0.0	0.0	18.0	1.0	0.0	0.0	0.0	89.5	4.0	13.5	9.0	11.5	20.0	19.0

* Oocyst count per loopful.[1] † Each number represents 19 fecal samples obtained from the first day of the inoculation until the twenty sixth day.

Necropsy Findings, Test 2.—Calf 1382 (Eimeria) was very dehydrated from scouring severely for several days before death on test day 26. Dehydration was also indicated by an increase in packed erythrocyte volume on the third day before death. The ileum was hyperemic and was swollen in scattered areas. The mucosa of the cecum, colon, and rectum was thickened and contained darkened ridges characteristic of clinical coccidiosis in cattle.[7] Fresh smears revealed the presence of myriads of merozoites in the ileum, cecum, and colon. No oocysts were found, but the enormous numbers of merozoites would indicate that a massive infection was underway.

Calf 1379 (Ostertagia) died on post inoculation day 30. The abomasum was inflamed and edematous, with widely distributed nodules in the mucosa. Some regions were eroded. In addition to the acute gastritis, there was pneumonic involvement of the apices of the lungs, the gallbladder was abnormally distended, and the small intestine was bile-stained. The abomasum of this animal was digested by the technique described by Herlich,[8] and almost 25,000 worms were recovered—all but 100 of them sexually mature. The total number represented about 10 per cent of the infective larvae administered.

Calf 1383 (Eimeria-Ostertagia) was killed while moribund on the thirty-ninth day. The lungs were discolored and were so congested that some portions would not float in water. The dry contents of the rumen and the hard fecal masses in the colon and rectum signified stasis of the digestive tract. The mesenteries were edematous. The nematodes had heavily damaged the abomasum, in which there was one ulcerous, crateriform lesion about 8 by 15 mm. in size. Small numbers of merozoites were seen in the posterior 18 feet of the small intestine. No other coccidia were seen, probably because the inoculated ones had completed their cycle and had been expelled. Slightly more than 8,000 worms were recovered—about 7 per cent of the infecting dose. All were mature, including 400 recovered after a portion of the abomasum was macerated in a Waring blendor. Again, digestion of the abomasum possibly would have resulted in the recovery of immature worms.

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Summary and Conclusions

To determine the effects on calves of single or concurrent infections with Eimeria spp. and *Ostertagia ostertagi*, 3 young calves in each of three groups were infected and 2 others were left as controls. One month after killing 1 calf from each test group on the twenty first day, the remaining calves were weaned and given challenge inoculations with the same organisms as before.

In the first test the only calf that developed an oocyst count greater than 10,000 per loopful, predominantly *Eimeria ellipsoidalis* on three consecutive days, was 1 of 3 inoculated with both Eimeria and Ostertagia. During the first 21 days the calves given Eimeria alone had diarrhea about twice as often as the other two parasitized groups combined. Weight gains of the infected calves were comparatively low during the first three weeks.

In the second test only 1 calf, given Eimeria only, had an oocyst count as great as 10,000 per loopful and it was due to *Eimeria alabamensis*. Calves in the Eimeria and the Eimeria-Ostertagia groups were the only ones that had diarrhea, and they showed it for an average of 5.5 and 6.0 days, respectively. The Ostertagia-infected calves gained an average of 19.0 lb. in 21 days, while calves in the other infected groups lost from 0.5 to 1.5 lb. The younger of the remaining calves in the parasitized groups died between post challenge days 26 and 39. Clinical signs of parasitism were more evident in the Eimeria-infected than in the Ostertagia-infected calves. The combination of the Eimeria attacking the intestine and Ostertagia attacking the abomasum concurrently apparently did not enhance the development of either parasite.

References

[1] Boughton, D. C.: Sulfaguanidine Therapy in Experimental Bovine Coccidiosis. Am. J. Vet. Res., *4*, (Jan., 1943): 66–72.

[2] Davis, L. R., Boughton, D. C., and Bowman, G. W.: Biology and Pathogenicity of *Eimeria Alabamensis* Christensen, 1941, an Intranuclear Coccidium of Cattle. Am. J. Vet. Res., *16*, (April, 1955): 274–281.

[3] Davis, L. R., and Bowman, G. W.: Coccidiosis in Cattle. Proc. U.S. Livestock San. A. (1951): 39–50.

[4] Davis, L. R., and Bowman, G. W.: The Endogenous Development of *Eimeria Zurnii*, a Pathogenic Coccidium of Cattle Am. J. Vet. Res., *18*, (July, 1957): 569–574.

[5] Davis, L. R., Bowman, G. W., and Porter, D. A.: Portable Pens Compared with Other Enclosures for Control of Diseases of Dairy Calves. Vet. Med., *47*, (1952): 485–490.

[6] Davis, L. R., Herlich, H., and Bowman, G. W.: Studies on Experimental Concurrent Infections of Dairy Calves with Coccidia and Nematodes. I. Eimeria spp. and the Small Intestinal Worm, *Cooperia Punctata*. Am. J. Vet. Res., *20*, (Jan., 1959): 281–286.

[7] Hammond, D. M., Davis, L. R., and Bowman, G. W.: Experimental Infections with *Eimeria Bovis* in Calves. Am. J. Vet. Res., *5*, (Oct., 1944): 303–311.

[8] Herlich, H.: A Digestion Method of Post-mortem Recovery of Nematodes from Ruminants. Proc. Helminthol. Soc. Washington, *23*, (1956): 102–103.

[9] Porter, D. A.: Incidence of Gastrointestinal Nematodes of Cattle in the Southeastern United States. Am. J. Vet. Res., *3*, (July, 1942): 304–308).

[10] Rohrbacher, G. H., Jr., Porter, D. A., and Herlich, H.: The Effect of Milk in the Diet of Calves and Rabbits Upon the Development of Trichostrongylid Nematodes. Am. J. Vet. Res., *19*, (July, 1958): 625–631.

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Studies on Bovine Gastrointestinal Parasites. XX. The Results of Feeding Small Amounts of Phenothiazine in Pure Infections of *Cooperia Punctata*

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SHORB AND HABERMAN [10] and Haberman and Shorb [3] failed to recover infective larvae from mixed natural infections in sheep as a result of feeding small amounts of phenothiazine. Mayhew [5] reported that 1.5 and 0.5 Gm. of phenothiazine was effective in the elimination of hookworm *(Bunostomum phlebotomum)* larvae,[6,9] and in eliminating the eggs of the nodular worm *(Oesophagostomum radiatum)* in pure infections. He reported [8] that feeding small amounts of phenothiazine daily was useful in reducing the number of infective larvae about the premises. In this manner, protection against infection is afforded. Mayhew [7] reported a reduction in egg output of the stomach worm *Haemonchus contortus* and Andrews *et al.*[1] a reduction of egg output in mixed infections. Cauthen [2] reported a reduction of infective larvae in mixed infections as a result of feeding phenothiazine.

The effect of phenothiazine fed in small amounts to animals with pure infections of *Cooperia punctata* is reported in the present study.

MATERIALS AND METHODS

The calves used in these experiments were of dairy types and mixed breeds and were obtained when 1 to 3 days old. They were confined in cages with iron-grating floors raised above the cement floor of the building to facilitate cleaning. The cages and floor were cleaned and washed with cold water followed by steaming hot water three times a week to prevent infection from the cages. The animals were inoculated with infective larvae from a pure infection of *Cooperia punctata* established in 1955.*

Cultures for larvae to inoculate calves and to determine the effectiveness of the phenothiazine were made as follows: approximately 4 lb. of fresh manure was placed in wire baskets, 12 inches square by 2 inches deep and incubated at 70 F. for 12 to 15 days, then placed in Baerman funnels overnight. To make the larvae counts, 50-cc. round-bottomed centrifuge tubes were filled with water from the funnels and the larvae allowed to settle 12 to 24 hours. The water then was carefully decanted to 15 to 20 cc., so as not to disturb the larvae.

During the feeding of phenothiazine, larvae counts were made by taking 2 drops from the bottom of the tubes for examination under the microscope. When phenothiazine was not being fed, so many larvae were present that it was necessary to put the larvae in suspension and use 2 drops to make even a rough estimate. When the drug was being fed, the number of larvae per culture usually varied from 0 to 48 (only 1 had 88) and when it was not being fed, the estimate ranged from 2,000 to 400,000 infective larvae per culture.

RESULTS

EXPERIMENTS USING 1.5 TO 12.0 GM. OF PHENOTHIAZINE PER DAY

Calf 275.—Two cultures made between April 13 and 16, 1955, yielded large numbers of larvae (fig. 1A ∞ –2). For the next 16 days, 1.5 Gm. of phenothiazine was fed daily and only a few larvae were recovered from four cultures (fig. 1A, Few–4), while four cultures made eight to 22 days after the feeding of the drug was stopped yielded large numbers of larvae (∞–2, ∞–2, fig. 1A; the bar indicates the days on which the cultures were made). Since the egg counts were not affected by the drug, larvae should have been recovered during the drug-feeding period the same as before and after feeding.

Additional low-level feeding experiments were carried out with calf 275 as indicated (fig. 1A, 1B). Few larvae were recovered during the periods when phenothiazine was being fed, while in each instance large numbers were recovered when the drug was not fed. In one instance (Aug. 6 to 20), 391 larvae were recovered from 12 cultures (an average of 33 per culture), a poor yield from approximately 50 lb. of manure as compared with 2,000 to 400,000 per culture from 4 lb. when the drug was not fed.

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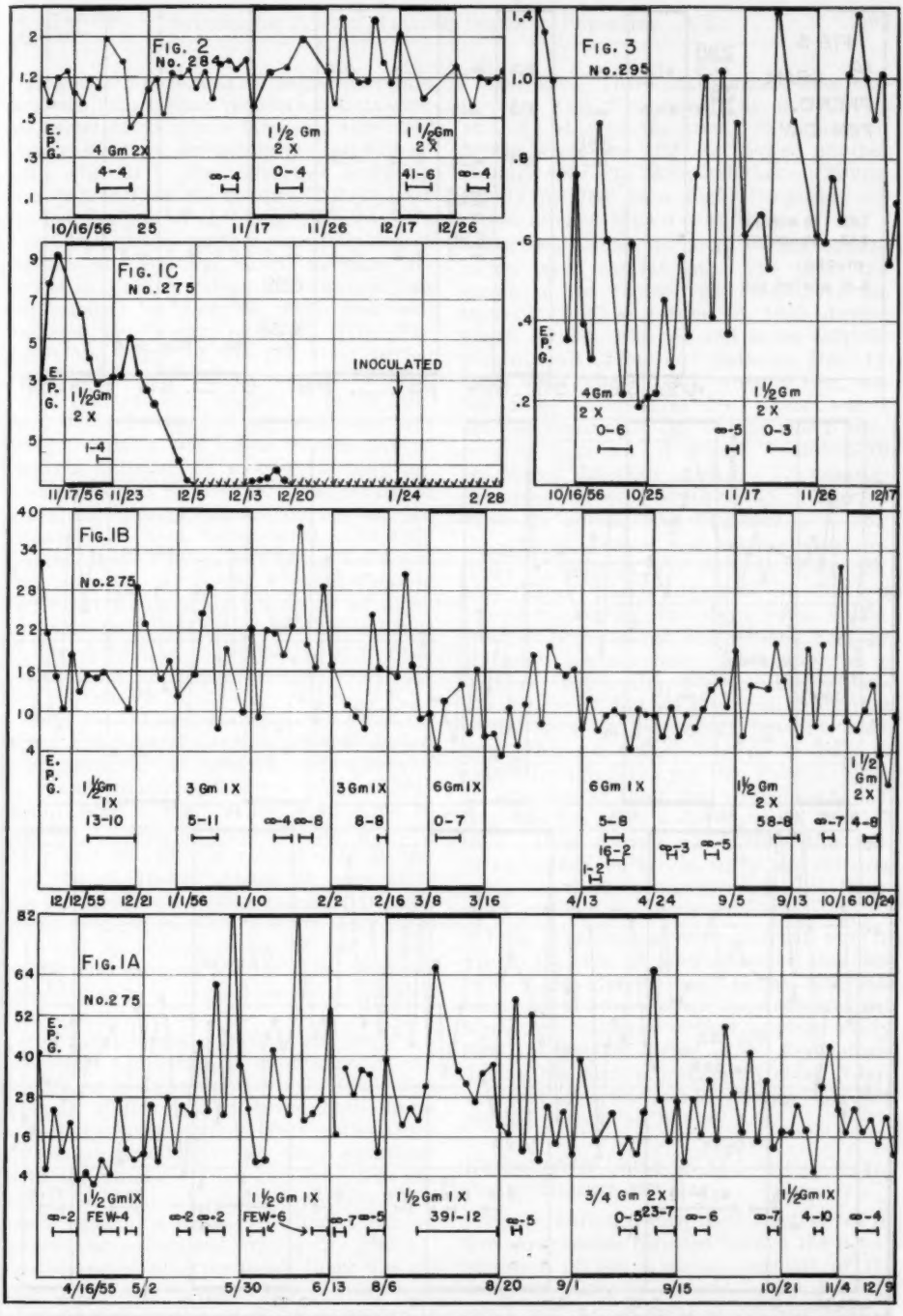
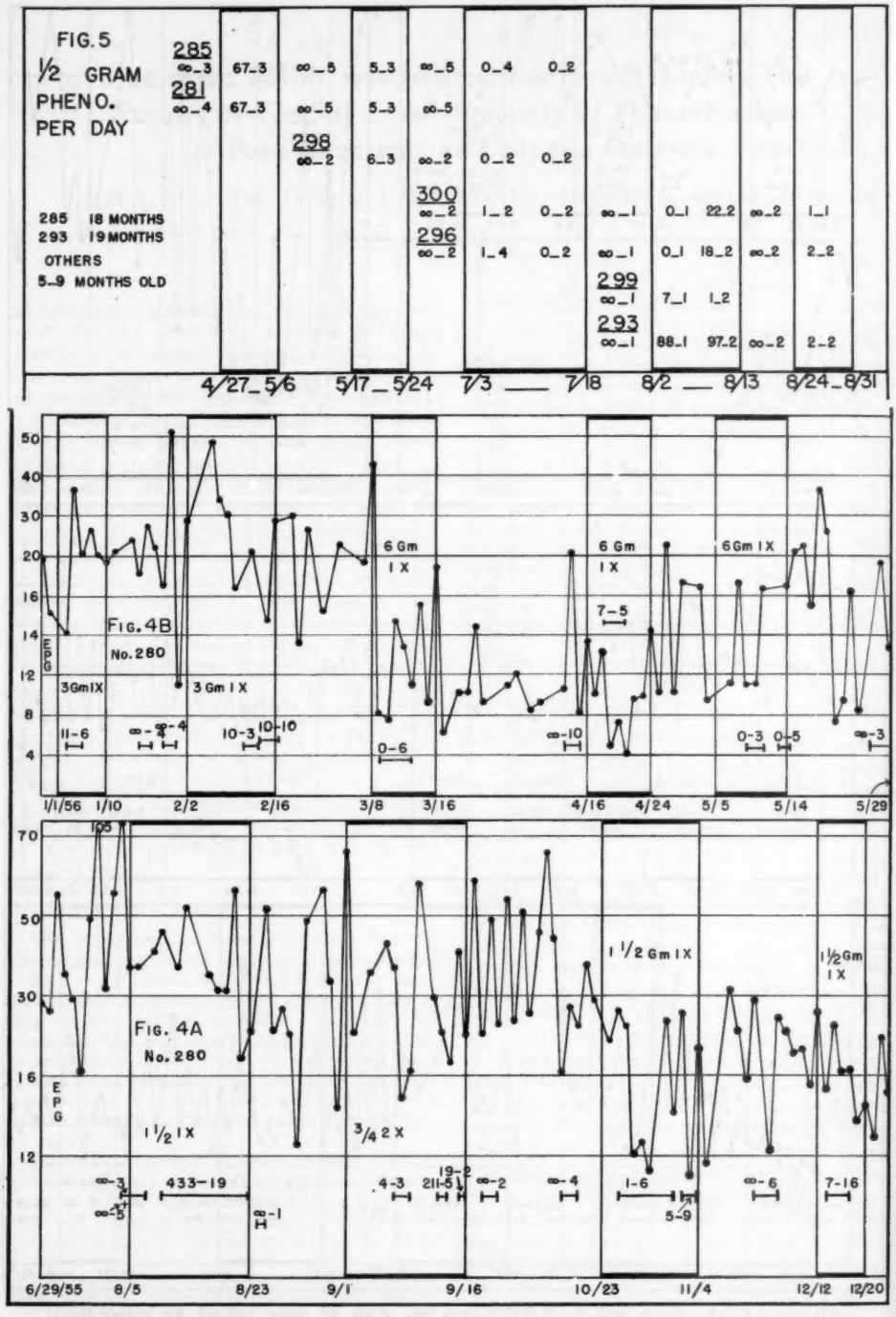
* Identification confirmed by Dr. Allen McIntosh, parasitologist, Animal Disease and Research Branch, U.S.D.A.



Figures 1A, 1B, 1C, 2, and 3—E.P.G. = eggs per gram of feces (shown by graph line); 1X, 2X = phenothiazine fed once or twice a day during period shown in blocked area; ∞ = sign of infinity, indicating large numbers of larvae recoveerd when phenothiazine was not being fed; 10-3, 0-7, 5-8, etc. = total number of larvae, followed by number of cultures taken during the period indicated by the bar (|——|).



Figures 4A, 4B, and 5—E.P.G. = eggs per gram of feces (shown by graph line: 1X, 2X = phenothiazine fed once or twice a day during period shown in blocked area; ∞ = sign of infinity, indicating large numbers of larvae recovered when phenothiazine was not being fed; 10-3, 0-7, 5-8, etc. = total number of larvae, following by number of cultures taken during the period indicated by the bar (|——|).

In an attempt to find a dosage that would remove all the larvae, various amounts were fed as indicated. None, however, were completely effective. Following the last experiment (fig. 1C), Cooperia eggs suddenly disappeared from the manure 12 days after the last feeding of the drug. We are confident that this is another manifestation of the immune reaction which we have observed so often in calves. This animal was reinoculated on Jan. 24, 1957, and still was negative 35 days later (fig. 1C). The maximum length of the prepatent period for *C. punctata* is approximately 14 days.

Calf 280.—From August 5 to 23 (fig. 4A), while 1.5 Gm. of phenothiazine was being fed daily, 433 larvae were recovered from 19 cultures, an average of only approximately 23 per culture. The results of additional experiments are shown (fig. 4A, 4B, and 6). From September 3 to 13, 1956 (fig. 6), while 6.0 Gm. was being fed night and morning, there was a rapid drop in egg count followed by a number of negative findings. That this is another instance of the development of immunity is indicated by the low egg count after reinoculation on October 1.

Calf 285.—Fecal examinations made between Feb. 17 and June 25, 1956, indicated a constant level of infection with yields of enormous numbers of larvae between May 9 and June 8 (fig. 7). Between June 16 and 25, 1.5 Gm. of phenothiazine was fed daily and six cultures yielded only 15 larvae (fig. 7, 15–6). About 21 days after the last feeding of phenothiazine, the egg count dropped rapidly to 0 for seven days followed by a long period of low counts with a maximum of 9 e.p.g. This was considered to be a typical immune reaction that was incomplete.

Calf 284.—Fecal examinations showed a consistent egg count of approximately 0.5 to 2.0 e.p.g. between Sept. 1, 1956, and Feb. 15, 1957 (fig. 2). However, the number of larvae recovered during the three treatment periods were low when compared with the numbers when the drug was not being fed. One additional experiment was made using this animal in which 6 Gm. of phenothiazine was fed twice a day (fig. 8). In this repeated experiment, there was almost no change in egg counts, a sharp contrast to the results in three experiments when 6 Gm. was fed twice daily (fig. 6, 9, 10) and in which an immune reaction occurred.

Calf 295.—Three experiments were carried out with this animal (fig. 3, 9). Two (fig. 3) showed the usual reductions of larvae while one (fig. 9) showed another immune reaction. Reinoculation on February 17 resulted in a slight temporary increase in eggs, then a drop to 0.

Calf 270.—Two low-level feeding experiments were carried out. The first, not shown in the figures, was conducted between May 30 and June 13, 1955, during which 1.5 Gm. was fed and seven cultures yielded only 3 larvae. Between Dec. 14, 1956, and Feb. 9, 1957, when 6 Gm. was fed twice a day (fig. 10), there was a marked decrease in egg counts, which later became negative. Although reinoculated on March 12 (not shown), 17 negative examinations were obtained up to April 1 when the animal was discarded.

Calf 290.—Three experiments were carried out; the results of two are shown (fig. 11). There was a decrease in the egg counts during the last days, when 6 Gm. was fed twice a day, followed by 13 days of much lower counts. This appears to indicate that 6 Gm. twice a day reduces the egg count, but we prefer to think that it indicates an incomplete immune reaction. This was followed by an increase to approximately 10 e.p.g. by Feb. 1, 1957. In addition to these two experiments, 1.5 Gm. was fed twice a day between Nov. 17 to 23, 1956, during which time nine cultures yielded no larvae while six cultures made about three weeks after this treatment yielded enormous numbers.

Two experiments were carried out in which 1.5 Gm. of phenothiazine was fed twice a day for 12- and 14-day intervals each (not shown). When the drug was not being fed, cultures yielded approximately 2,000 to 300,000 larvae each. Four made during the first experiment yielded 5 larvae and the five made during the last yielded few to no larvae.

EXPERIMENTS USING 0.5 GM. OF PHENOTHIAZINE PER DAY

Seven animals were used in a series of five experiments directed toward the determination of the minimum amount of the drug necessary to prevent larvae development (fig. 5). A total of 227 egg counts, made daily except Saturdays and Sundays, are omitted but eggs were continuously demonstrated to be present. The older ani-

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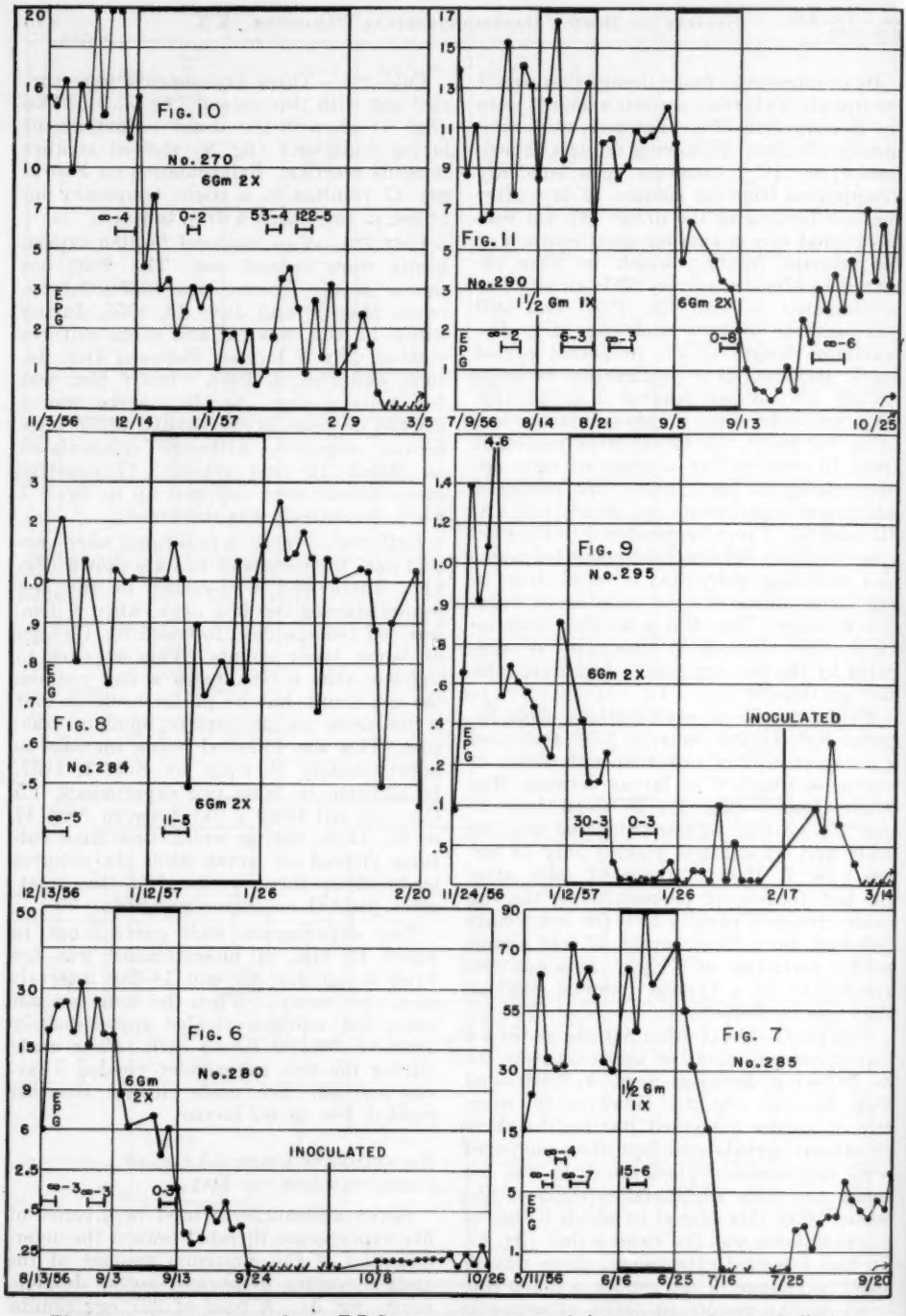

Figures 6, 7, 8, 9, 10, and 11—E.P.G. = eggs per gram of feces (shown by graph line); 1X, 2X = phenothiazine fed once or twice a day during period shown in blocked area; ∞ = sign of infinity, indicating large numbers of larvae recovered when phenothiazine was not being fed; 10-3, 0-7, 5-8, etc. = total number of larvae, followed by number of cultures taken during the period indicated by the bar (|——|).

mals (285, 18 months old, and 293, 19 months old) were selected to determine if age and size would influence the results. The others were 5 to 9 months old when first used. In figure 5, the dates of the experiments are at the bottom. As when larger amounts of the drug were fed, and regardless of the individual and its age, there was a definite lowering of the number of larvae recovered as compared to when it was not being fed. Two animals developed immune reactions associated with these 0.5 Gm. experiments.

SUMMARY

In 56 experiments on 14 cattle, it was found that daily feeding of small amounts of phenothiazine had no effect on egg production of *Cooperia punctata*, but was 100 per cent effective in reducing the number of infective larvae to a very low level.

The reduction of egg count levels in some individuals is believed to result from immune reactions rather than the drug, because there is no consistent relationship to the amounts being fed or to the time of the experiment. These animals also proved to be resistant to reinfection.

References

[1] Andrews, J. S., Muniz, C. M., and Orihuela, F.: Preliminary Note on the Administration of Nonconditioned Phenothiazine, in Small Daily Doses for the Control of Gastro-Intestinal Parasites of Cattle in Puerto Rico. J. Agric., Univ. Puerto Rico, *27*, (1944): 125–130.

[2] Cauthen, G. E.: The Effects of Small Daily Doses of Phenothiazine on the Development of Larvae of Gastro-Intestinal Parasites of Cattle. Am. J. Vet. Res., *14*, (Jan., 1953): 30–32.

[3] Habermann, R. T., and Shorb, D. A.: The Effects of Phenothiazine-Salt Mixtures on the Development of Parasitic Larvae in the Feces of Sheep. North Am. Vet., *23*, (1942): 318–321.

[4] Landram, J. F., and Cauthen, G. E.: Some Experiments on the Effect of Low Level Phenothiazine on the Development of *Ostertagia Ostertagi* Larvae in Bovine Feces. Proc. Helminthol. Soc. Washington, *23*, (1956): 24–25.

[5] Mayhew, R. L.: Studies on Bovine Gastro-Intestinal Parasites. XVI. Some Results of Feeding Small Amounts of Phenothiazine on Pure Infections of the Hookworm *Bunostomum Phlebotomum*. J. Parasitol., *36*, (1950): 536–540.

[6] Mayhew, R. L.: Studies on Bovine Gastro-Intestinal Parasites. XVIII. Some Results of Feeding Small Amounts of Phenothiazine on Pure Infections of the Nodular Worm *Oesophagostomum Radiatum*. Proc. Helminthol. Soc. Washington, *18*, (1951): 70–77.

[7] Mayhew, R. L.: Results of Feeding Small Amounts of Phenothiazine in Pure Infection of the Large Stomach Worm *Haemonchus Contortus*. J. Parasitol., *38*, (1952): 30–31.

[8] Mayhew, R. L.: The Parasites and Parasitic Diseases of Cattle. Louisiana Bull. No. 428 (1953): 46.

Mayhew, R. L.: Studies on Bovine Gastro-Intestinal Parasites. XIX. Further Studies in the Use of Small Amounts of Phenothiazine in Pure Infections of the Nodular Worm. J. Parasitol., *38*, (1952): 525–531.

[10] Shorb, D. A., and Habermann, R. J.: Effect of Phenothiazine on the Development of Roundworm Larvae in the Feces. Vet. Med., *35*, (1940): 454–457.

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Studies on Bovine Gastrointestinal Parasites. XXII. Rate of Consumption of Phenothiazine in Salt-Phenothiazine Mixtures by Cattle

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In 1947 it was decided to undertake a study of the amount of phenothiazine consumed "free choice" by cattle on pasture. This seemed desirable, since salt mixtures were being used to some extent by cattle owners to control parasites, although the practice was much more extensively used by sheep growers at the time. Since we had previously determined the amount of the drug necessary to control the development of larvae, the experiment was not one dealing with the control of parasites, but one in which it was desired to determine how much of the mixture healthy animals would consume on pasture.

The effects of low-level feeding of phenothiazine in the control of various parasites have been reported by several workers.[1-12] Reference to some of the most pertinent reports are given, but no attempt is made to give a complete list.

Materials and Methods

Two pastures of about 2 acres each were available. They had been used for the past six years for certain observations on animals that had been parasitized under controlled conditions. The 36 dairy-type cattle used in the salt-phenothiazine studies had been previously inoculated with *Haemonchus contortus* and *Oesophagostomum radiatum* under controlled conditions. These infections had disappeared or had become very low and the animals were in good condition when taken to the pastures.

The grasses were predominately Burmuda, with some carpet and Dallis grass and white and bur clover present in the spring. Hay was provided during dry periods and winter months. The cattle were provided with ample water and a small barn for shelter. During the years 1947 to 1949, the animals were in two groups; after September, 1950, each was provided with its own shelter, water supply, and about one third of an acre of pasture. The salt-phenothiazine mixture and pure granular salt was provided in 2.5-gal. buckets to the animals using the shelters, but the 2 animals having access to the barn obtained theirs from open wooden boxes (8 x 15 x 6 in.) inside the barn. The salt-phenothiazine mixture was in proportions of 10 to 1.

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Experimental Data

On June 20, 1947, the 4 cattle already in the pasture were separated by a partition in the barn so that 2 had access to each side. Salt only was provided from June 20 to September 25 (fig. 1). The salt remaining was removed, and a weighed amount of the salt placed in the box in one stall and an equal amount of the salt-phenothiazine mixture in the other. The 2 animals having access to the mixture consumed somewhat more of it (fig. 1) than they had of the pure salt previously. (The lower shaded portions of the columns indicate the average number of grams of phenothiazine consumed per day.) The 2 animals given pure salt consumed about twice as much as they had previously.

Observations were discontinued from Feb. 1 to May 1, 1948, when animal 170 was added to the experiment, and the salt-phenothiazine mixture was supplied to both groups. From then until August 23, the animals in both groups consumed considerably less of the mixture than previously (fig. 1).

All of these animals were then replaced, except 170, and the experiment was started again November 13, all 5 being given the mixture as a group (fig. 1). The average amount per animal per day until Feb. 24, 1949, was somewhat more than during the preceding period, and closely approximated that consumed between May 1 and July 14.

Two groups of 3 animals each were maintained between May 13, and Nov. 10, 1949 (fig. 1). One group was provided with the salt-phenothiazine mixture and the other group with salt. The groups given pure salt consumed more than any other group of animals during the entire experiment (fig. 1, 2, 3). Those given the salt-phenothiazine mixture consumed from 0.0 to 27.0 Gm. in the different time periods. The amount of phenothiazine consumed

was below the 0.5-Gm. minimum necessary for parasite control during some of the time periods.

Because of these observations, it was decided to keep, as nearly as possible, the same animals under continuous observation for longer periods and to isolate them as early as possible. An additional two thirds of an acre of pasture was made available adjoining the others. These objectives were not completely attained until Sept. 16, 1950.

On April 18, 1950, animals 196, 199, and 201 in one pasture and 191 in the new pasture were provided with the mixture, while animals 198 and 200 in the other pasture were given salt only (fig. 2). This was maintained until September 2, when, with additional facilities completed, the animals given the salt-phenothiazine mixture were separated. On September 16, the salt-fed animals were likewise separated. One of the 2 animals that utilized the barn with the original salt boxes was offered salt and the other was offered the mixture. The other cattle obtained salt or the salt-phenothiazine mixture from 2.5-gal. buckets. The type of container apparently did not affect the amount consumed.

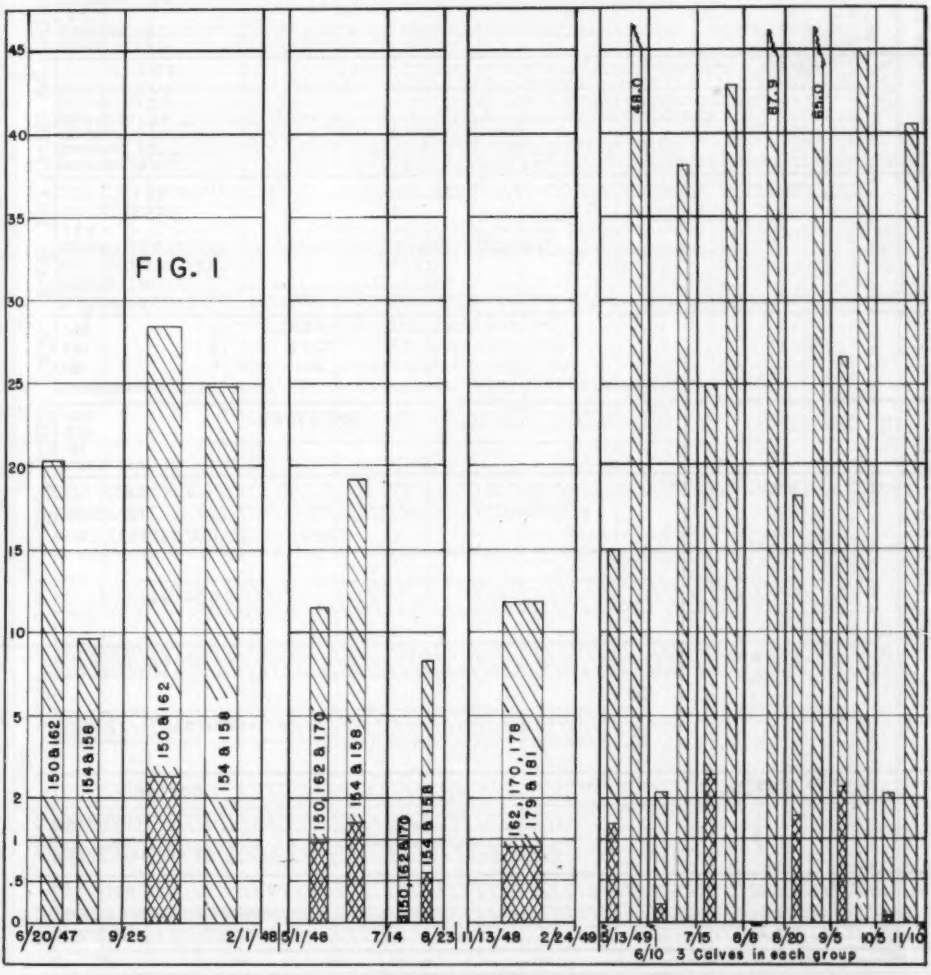

Fig. 1—Grams of salt or salt plus phenothiazine mixture (drug shown by shaded portion at base of bar) consumed per day (average for the period) by the animal indicated by numbers on the bar.

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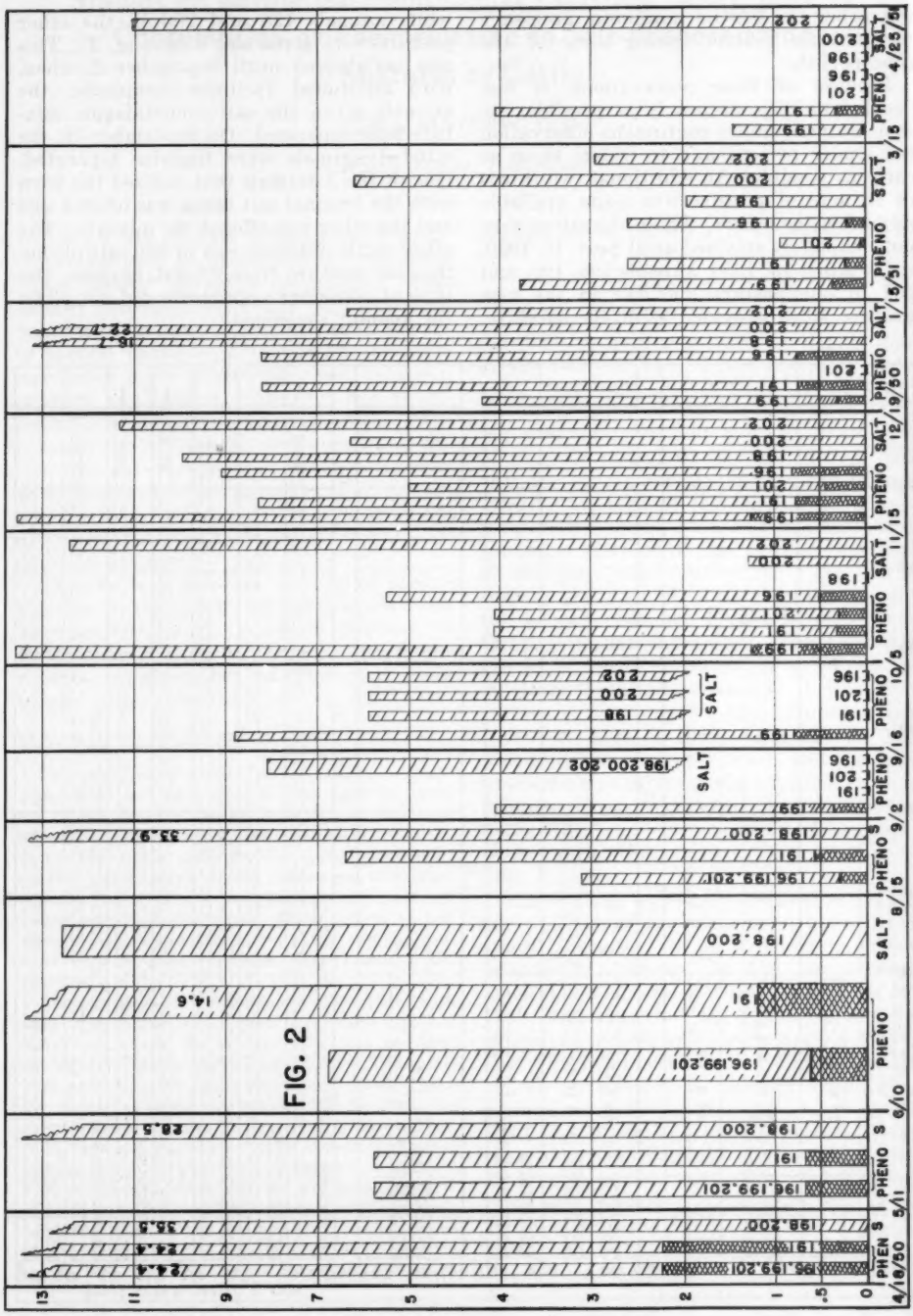

Fig. 2—Average grams of salt or salt plus phenothiazine consumed per day by animals indicated by numbers, for various periods during a year (1950–1951).

Fig. 2—Average grams of salt or salt plus phenothiazine consumed per day by animals indicated by numbers, for various periods during a year (1950–1951).

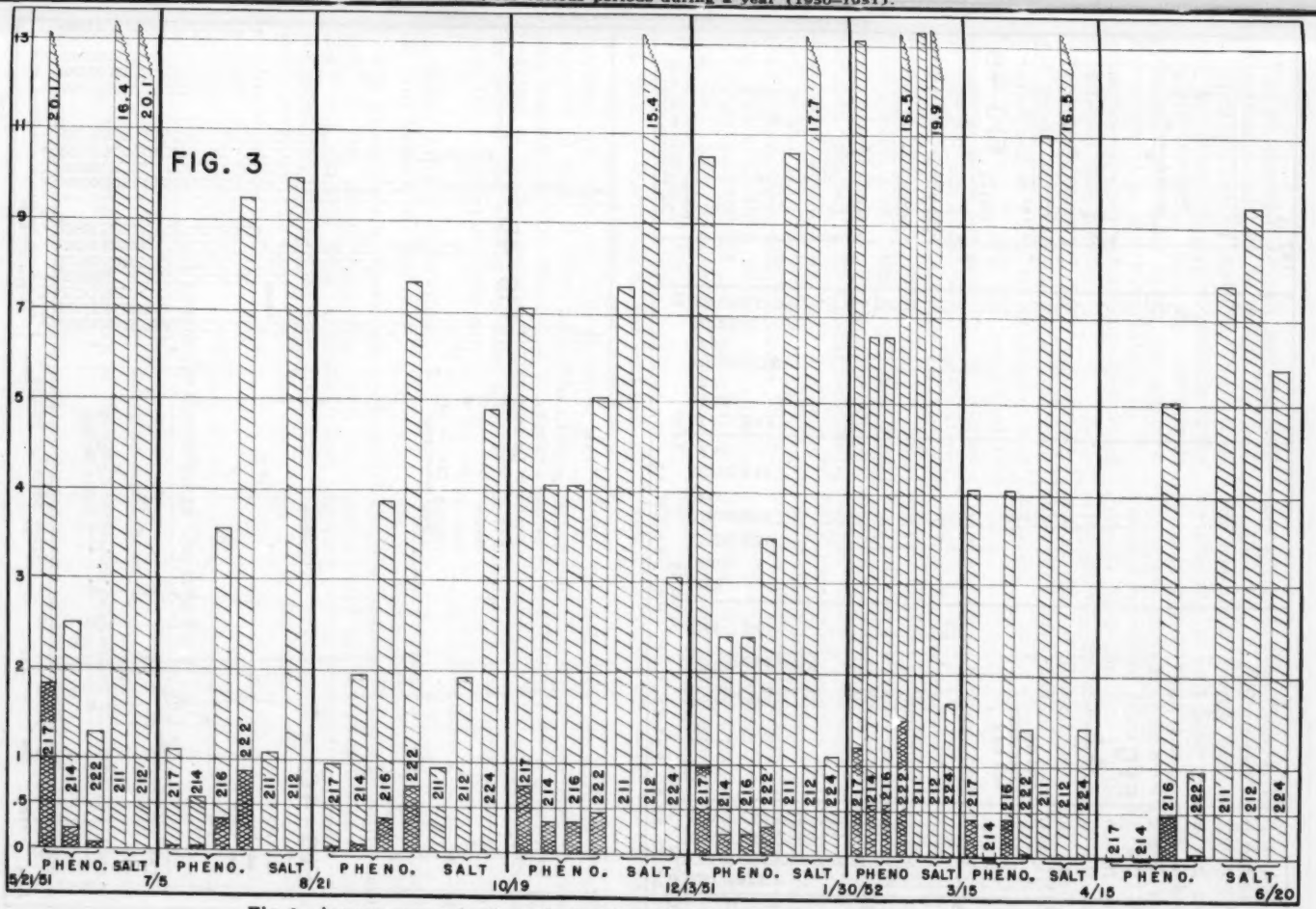

Fig. 3—Average grams of salt or salt plus phenothiazine consumed per day by animals indicated by numbers, for various periods during a year (1951–1952).

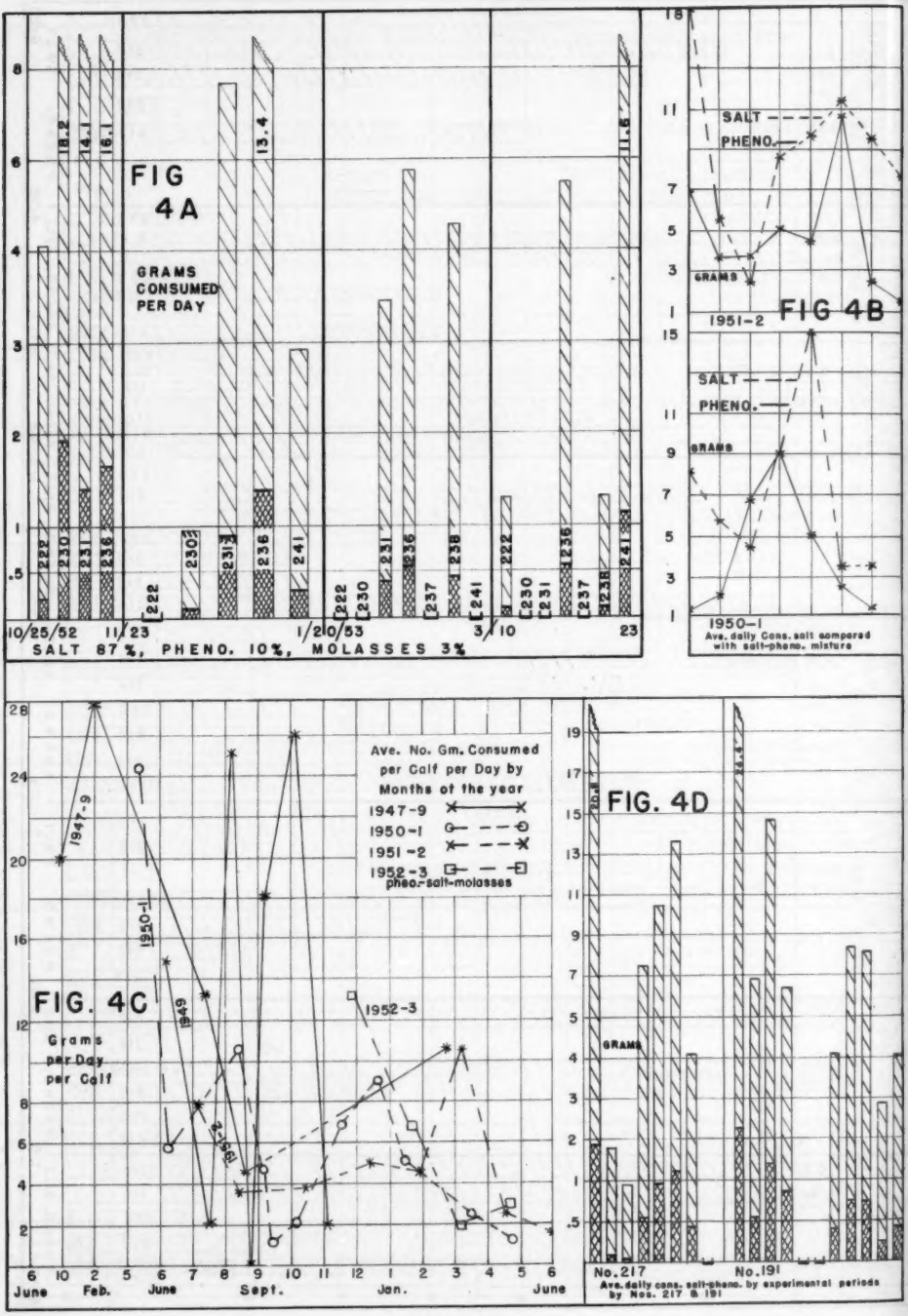
FIG 4A
GRAMS CONSUMED PER DAY
18.2
14.1
16.1
13.4
11.6
10/25/52
11/23
1/20/53
3/10
23
SALT 87%, PHENO. 10%, MOLASSES 3%
FIG 4B
SALT
PHENO.
GRAMS
1951-2
1950-1
Ave. daily Cons. salt compared with salt-pheno. mixture
FIG. 4C
Grams per Day per Calf
Ave. No. Gm. Consumed per Calf per Day by Months of the year
1947-9
1950-1
1951-2
1952-3
pheo.-salt-molasses
1947-9
1950-1
1949
1952-3
June
Feb.
June
Sept.
Jan.
June
FIG. 4D
GRAMS
No. 217
No. 191
Ave. daily cons. salt-pheno. by experimental periods by Nos. 217 & 191

Figure 4

The consumption of salt and the salt-phenothiazine mixture from Sept. 16, 1950 to April 25, 1951 is shown by individuals (fig. 2). There was a great variation in the amounts consumed both by individuals given salt and those given the mixture. There was also much variation in the amounts consumed by the same individual in different time periods.

Beginning May 21, 1951, an entirely new group of 5 animals was used, salt being offered to 2 and the mixture to 3 of them. On July 5, calf 216 was added to the group given the mixture; on August 21, calf 224 was added to the group given pure salt. This arrangement was maintained until June 20, 1952 (fig. 3). The same general conclusions were reached as in the case of the preceding animals. Much variation occurred in the amount consumed among individuals in the same time period and in the same individual in different time periods. Also, except between Jan. 30 and March 15, 1952, most of the animals given the mixture did not obtain enough (0.5 Gm.) phenothiazine to effectively control the parasitic larvae.

In October, 1952, a supply of a commercial mixture containing 87 per cent salt, 10 per cent phenothiazine, and 3 per cent molasses was obtained. The molasses was expected to make it more palatable. This was fed (fig. 4A) to animals in the same quarters as in the preceding experiments. In general, the results were the same as with the salt-phenothiazine only.

Average Daily Consumption of Salt Versus the Mixture

The average daily consumption of salt and of the salt-phenothiazine mixture, after the animals were separated, was compared by data periods (fig. 4B). The lower portion of the figure shows the averages consumed from Sept. 2, 1950 to April 25, 1951 (fig. 2). More salt than mixture was consumed during five of the seven periods, and in only four periods did the amounts closely approximate each other. The upper portion of figure 4B shows the averages consumed from May 21, 1951 to June 20, 1952 (fig. 3). During five of the seven periods the consumption of salt exceeded that of the mixture, and during the other two periods there was little difference.

Consumption by Seasons of the Year

When the consumption of the salt-phenothiazine mixture is compared by seasons of the year (fig. 4C), most of the groups of animals consumed less during the fall months. These data indicate no clearly defined seasonal variation in the consumption of the mixture. Much of the time it was below 5.0 Gm. of the mixture, the amount necessary to supply the amount of the drug (0.5 Gm.) required to control larval development. Some groups of animals consumed larger amounts when it was first offered than they did later, but this was not true of the animals in the 1949 and 1951–1952 groups.

The variability in the amount of salt-phenothiazine mixture consumed by animals 217 and 191 in various seasons of the year is shown (fig. 4D) as taken from figures 2 and 3.

Summary

Data are presented on the average daily rate of consumption of salt and of a 10:1 salt-phenothiazine mixture by cattle maintained under the same conditions of management over a period of five years. Animals were individually isolated during 21 months of this time.

Legend for Illustration on Opposite Page

Fig. 4A—Average grams of salt-phenothiazine-molasses mixture consumed per animal per day during the period shown.

Fig. 4B—Comparison of the salt vs. the salt-phenothiazine consumption as summarized from figures 2 and 3.

Fig. 4C—Seasonal variation in the salt-phenothiazine mixture consumed by the groups of calves during different years.

Fig. 4D—Average daily consumption of salt and phenothiazine, by calves 217 and 191, at various seasons in one year.

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There was a wide variation in the amounts of salt and of the salt-phenothiazine mixture consumed by different individuals in the same period and by the same individual in different periods.

The amount of the mixture consumed was often less than that necessary to supply the 0.5 Gm. of the drug necessary to control larvae development, indicating that a salt-phenothiazine mixture is not a reliable method of administering the drug for maximum effectiveness.

A commercial salt-phenothiazine-molasses mixture was not consumed more consistently or readily than was the salt-phenothiazine alone.

There was no consistent difference in the rate of consumption of the salt-phenothiazine mixture during different periods or seasons of the year.

References

[1] Andrews, J. S., Muniz, C. M., and Oriheula, F.: Preliminary Note on the Administration of Non-Conditioned Phenothiazine, in Small Daily Doses, for the Control of Gastro-Intestinal Parasites of Cattle in Puerto Rico. J. Agric., Univ. Puerto Rico, *27*, (1943): 125–130.

[2] Britton, J. W., Miller, R. F., and Cameron, H. S.: Phenothiazine-Salt Mixtures in the Control of Parasitism. Cornell Vet., *33*, (1943): 339–343

[3] Cauthen, George E.: The Effects of Small Daily Doses of Phenothiazine on the Development of Larvae of the Gastro-Intestinal Parasites of Cattle. Am. J. Vet. Res., *14*, (Jan., 1953): 30–32.

[4] Haberman, R. J.: Weekly "Salting" with Phenothiazine to Control Parasites. Vet. Med., *40*, (1945): 231–234.

[5] Harwood, P. D., Guthrie, J. E., and Preble, N. A.: Phenothiazine-Salt Mixtures for the Control of Gastro-Intestinal Parasites of Beef Cattle on Pasture. J. Tennessee Acad. Sci., *20*, (1945): 159–173.

[6] Herlich, Harry, and Porter, Dale A.: Control of Internal Parasites of Cattle by Free-Choice Administration of Phenothiazine. Vet. Med., *49*, (1954): 103–106.

[7] Landram, James F., and Cauthen, George E.: Some Experiments on the Effects of Low Level Phenothiazine on the Development of *Ostertagia Ostertagi* Larvae in Bovine Feces. Proc. Helminthol. Soc. Washington, *23*, (1956): 24–25.

[8] Mayhew, Roy L.: Studies on Bovine Gastro-Intestinal Parasites. XVI. Some Results of Feeding Small Amounts of Phenothiazine on Pure Infections of the Hookworm *Bunostomum Phlebotomum*. J. Parasitol., *36*, (1950): 536–540.

[9] Mayhew, Roy L.: Studies on Bovine Gastro-Intestinal Parasites. XVIII. Some Results of Feeding Small Amounts of Phenothiazine on Pure Infections of the Nodular Worm *Oesophagostomum Radiatum*. Proc. Helminthol. Soc. Washington, *18*, (1951): 70–77.

[10] Mayhew, Roy L.: Studies on Bovine Gastro-Intestinal Parasites. XIX. Further Studies in the Use of Small Amounts of Phenothiazine in Pure Infections of the Nodular Worm. J. Parasitol., *38*, (1952): 525–531.

[11] Mayhew, Roy L.: Results of Feeding Small Amounts of Phenothiazine in Pure Infections of the Large Stomach Worm *Haemonchus Contortus* in the Calf. J. Parasitol., *38*, (1952): 30–31.

[12] Shorb, D. A., and Haberman, Robert T.: Effect of Phenothiazine on the Development of Roundworm Larvae in the Feces. Vet. Med., *35*, (1940): 454–457.

Infectious Bovine Rhinotracheitis—The Occurrence of Intranuclear Inclusion Bodies in Experimentally Infected Animals

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SINCE the first descriptions of infectious bovine rhinotracheitis (IBR), several papers have dealt in part with the etiology and pathology of the disease.[6,9,11] In these reports, it was either stated that no inclusion bodies were found[7,8] or no mention was made of their occurrence.[3]

McKercher *et al.*[8] reported that IBR is, in most cases, manifested as a mild inflamation of the upper respiratory tract. These authors stated that the more extensive lesions undoubtedly developed as a result of bacterial invasion and the pure viral infection was probably nothing more than acute catarrhal inflammation.

Following the report of the cultivation of the causative agent of this disease in tissue culture by Madin *et al.*,[5] we were able to demonstrate that intranuclear inclusion bodies were a constant feature of the cytopathogenicity of several strains of the virus in tissue cultures of bovine kidney and human amnion.[1] With this demonstration, it seemed desirable to reinvestigate the histopathology of the disease, with particular reference to the occurrence of inclusion bodies in the natural host under experimental conditions.

This paper describes the inclusion bodies as they appear in the experimentally infected calves and reports several conditions which influence their demonstration.

MATERIALS AND METHODS

Tissue Cultures.—Cultures of young, adult, bovine kidney cells were prepared by the trypsin-digestion method of Madin.[4] Nutrient medium consisted of 95 per cent lactalbumin hydrolysate-Tris solution [0.5% lactalbumin hydrolysate in Hanks' balanced salt solution, modified by the addition of 0.01 M Tris (hydroxymethyl aminomethane) adjusted to a pH of 7.4] and 5 per cent lamb serum. Phenol red (0.002%), penicillin (500 units/ml.), streptomycin (0.5 mg./ml.), and myeostatin (100 units/ml.) were incorporated in the medium.

Virus.—Three strains of virus were studied. The first was provided as fluid from the fifteenth tissue culture passage and is referred to as the Pritchard strain.* The American and Cooper strains were obtained as nasal washings from animals with clinical cases of IBR.† Cytopathogenic agents were isolated and passed serially from both nasal washings in cultures of bovine kidney cells. The 50 per cent infective dose ($t.c.i.d._{50}$) was calculated by the method of Reed and Muench[10] and was based on an inoculum of 0.1 ml. per culture tube.

Experimental Animals.—Nine dairy calves, 4 to 5 months old, and 1 Hereford calf, 4 months old, were used. All calves were obtained locally from farms on which IBR had not been encountered. The animals were allowed to adjust to their new environment indoors without handling for 12 days. During the next two weeks, rectal temperatures and leukocyte counts were taken daily and each animal was observed for signs of illness. All temperatures and leukocyte counts were considered to be within normal limits and the animals appeared healthy.

During this observation period, nasal smears were taken and assayed for the presence of cytopathogenic agents in tissue culture; no cytopathogenic agents were isolated. Blood samples were taken from all the animals and serum-neutralization tests were performed, utilizing serum dilutions against 100 $t.c.i.d._{50}$ doses of the American strain virus in tissue cultures of bovine kidney. All serums were negative for antibodies for IBR virus, as evidenced by their failure to inhibit cytopathogenic changes in tissue culture in the lowest dilution tested (1:2).

Nasal secretions were collected prior to inoculation and again at the time of necropsy by placing sterile cotton swabs in each nostril, streaking across the septum and turbinates, and immersing in 1 ml. of tissue culture medium. Nasal specimens and portions of lung taken at the time of necropsy were stored at −60 C. until assayed for virus.

Animal Inoculation.—Calves were inoculated with 5 ml. of undiluted, infected, tissue culture fluid by intranasal instillation with a nasal atomizer. In addition to intranasal inoculation, calf 65 was given 5 ml. intratracheally. Each inoculum of 5 ml. was calculated to contain approximately 6.75

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* Supplied by Dr. W. R. Pritchard, Gainesville, Fla.
† Obtained from Dr. T. L. Chow, Colorado State University, Fort Collins.

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Fig. 1—Calf 65 showing nasal discharge and slight lacrimation 55 hours following inoculation with infectious bovine rhinotracheitis virus.

$\times 10^7$ t.c.i.d.$_{50}$. Seven calves were given the third tissue culture passage of the American strain virus. Calf 82 was given the second tissue culture passage of the Cooper strain, and calf 83 was given the nineteenth tissue culture passage of the Pritchard strain virus.

Necropsy Protocol.—One calf was killed before inoculation as a normal control animal. The animals inoculated with Pritchard and Cooper strains were killed at 55 and 49 hours, respectively. The remaining animals were killed at 12, 23, 36, 48, 55, 60, and 71 hours following inoculation with the American strain. With 1 exception, the animals were killed by electrocution. One calf was given a lethal dose of sodium pentobarbitol. A complete necropsy was performed immediately after death. Representative tissues of all organs were fixed in Bouin's fluid. Sections were stained with hematoxylin and eosin.

RESULTS

The clinical response of the inoculated animals was similar to that as reported by other authors.[7,8] The animals generally displayed sudden onset of illness with elevation of temperature, salivation, slight serous nasal discharge, and lacrimation (fig. 1). The clinical signs observed in each animal and the results of virus isolations are summarized (table 1).

Gross Pathology.—The most significant lesions developed in the upper respiratory tract, particularly in the mucosa of the nasal passages, turbinates, and paranasal sinuses (fig. 2). With the American strain of virus, examination of these areas revealed numerous petechiae and granularity of the mucosa as early as 12 hours after inoculation. Congestion and serous exudate was present after 36 hours. At 48 hours, the exudate was mucoid in character, and by 71 hours there were focal superficial ulcerations of the mucosa and abundant mucopurulent exudate. Some animals also showed

TABLE 1—Clinical Response in 9 Calves Inoculated with IBR Virus

Calf No.	Virus	Clinical symptoms	Virus recovery		Temperature recordings				
						Postinoculation			
			Nasal	Lung	Pre-inoc.	Highest	Time (hr.)	At necropsy	Time (hr.)
82	C	Slight conjunctival congestion with some lacrimation. Mucous exudate around external nares	N.A.*	N.A.	102.2	106.0	43	105.6	49
65	A	Mild diarrhea with some mucus in feces. Salivation and lacrimation.	+	N.A.	101.8	105.4	53	104.6	55
83	P	Minimal conjunctival congestion with slight serous exudate, copious amount of mucous exudate about external nares.	+	—	101.8	103.4	41	103.0	55
78	A	None	+	—	102.0	101.8	12	101.8	12
80	A	No signs of illness other than mildly forced abdominal breathing	+	—	102.2	103.4	23	103.4	23
79	A	Conjunctival congestion with serous lacrimation and nasal discharge, depressed, deep dry cough.	+	—	102.0	106.1	36	106.1	36
77	A	Very depressed, dry, deep, painful cough.	+	+	102.0	106.2	48	106.2	48
85	A	Conjunctival congestion with profuse lacrimation and seropurulent discharge. Mucopurulent discharge from nostrils.	+	+	101.2	105.0	60	105.0	60
81	A	Slight depression, serous discharge from eyes and nostrils.	—	—	102.1	103.9	71	103.9	71

Virus code: A = American virus; C = Cooper virus; P = Pritchard virus; * N.A. = not attempted.

mild congestion of the mucosa of the larynx, trachea, and bronchi, and a few petechiae in the epiglottis.

Small areas of atelectasis or pneumonic consolidation and petechiae were seen in some animals. In the digestive tract, congestion of the mucosa became increasingly severe with time and, after the sixtieth hour, petechiae were also present. The lymph nodes were enlarged, bulged on cut surface, and revealed scattered petechiae. Other lesions observed were minor and inconsistent.

The lesions observed in the 2 calves inoculated with the Cooper and Pritchard viruses were similar to those of calves given the American strain of virus, when killed at comparable times.

Histopathology.—The salient cytological features appeared in the mucosa of the upper respiratory tract. Similar changes occurred in both stratified squamous and pseudostratified columnar epithelial cells. The cytoplasm of such cells was frequently pale and vacuolated. Later, it showed ballooning degeneration, became granular, and the cells lost their peripheral outline. Nuclear changes were observed in some of the cells showing early cytoplasmic alterations. Small, irregular aggregates of pale, acidophilic material were first noticed dispersed among the nuclear chromatin. Simultaneously, the chromatin became marginated and the nucleolus either fragmented and disappeared or contracted and remained at the nuclear margin as a small basophilic body. The pink material tended to become more homogeneous and filled the major portion of the nucleus. Later, this material contracted, leaving a clear halo surrounding a now distinct, acidophilic, and often irregular, inclusion body (fig. 3). These inclusions were seen throughout the mucosa and in cells of tissue fragments separated from the surface.

At 12 hours, the mucosa of the nasal septum, turbinates, and nictitating membrane presented a few foci of degenerating cells with early cytoplasmic changes, but without definite nuclear changes.

At the twenty-fourth hour, these lesions were more prominent, and the pharynx was similarly involved. Early nuclear changes appeared in the turbinates, but they did not progress to distinct inclusions at that time.

By 36 hours, numerous well-formed intranuclear inclusions were first seen in the epithelial cells of the nasal septum, and cytoplasmic changes occurred in epithelial cells of the trachea.

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Fig. 2—Turbinates and nasal septum of a calf 55 hours after inoculation showing petechiae and granularity of the mucosa.

At 48 and 60 hours, nuclear inclusions appeared in the turbinates and bronchi. Early nuclear changes became apparent in the trachea, but at no time were well-develop inclusions observed.

At 71 hours, the previously listed changes prevailed, with the exception of well-formed inclusions, which were not found.

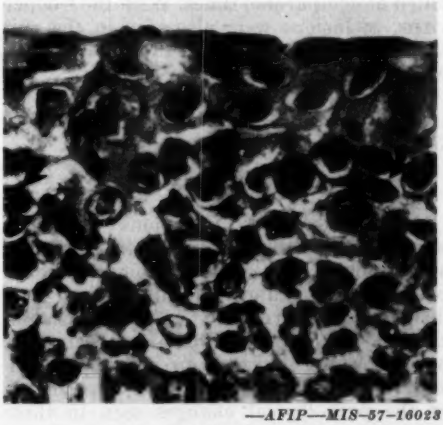

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Fig. 3—Intranuclear inclusion bodies (arrows) in epithelial cells of nasal septum of calf at 36 hours. H & E stain; x 700.

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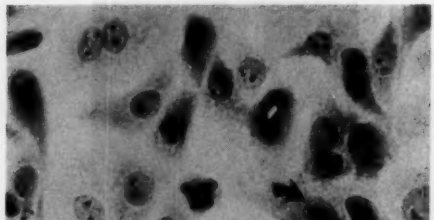

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Fig. 4—Intranuclear inclusion bodies (arrows) in tissue culture of bovine kidney cells infected with American strain of infectious bovine rhinotracheitis virus. H & E stain; x 315.

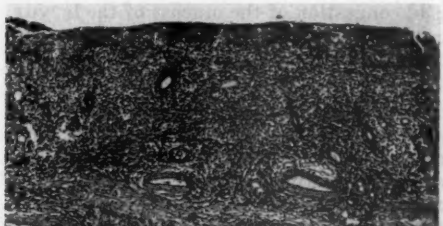

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Fig. 5—Same preparation as figure 3 showing inflammatory infiltrate. H & E stain; x 35.

When these specific cellular changes caused a break in the mucosal surface, they were accompanied by the development of an acute inflammatory infiltrate and secondary bacterial infection (fig. 5). In general, this sequence of cell degeneration, erosion, inflammation, and exudation increased in severity during the 12- to 71-hour period after inoculation.

With one exception, the histological lesions in the calves given the Cooper and Pritchard viruses were consistent with those given the American strain of virus, when killed at comparable times. With the Cooper virus, inclusions were also seen in the epithelial cells of the pharynx in the calf which was killed at 49 hours.

Lesions in other tissues were not indicative of the viral activity and were considered secondary in nature.

DISCUSSION

As reported here, inclusions were first seen in cells of the upper respiratory tract at 36 hours, and persisted through 60 hours postinfection. Since the inclusions were demonstrated primarily in the epithelial cells of the upper respiratory tract, it is not surprising that their presence was transitory in nature. Although a major portion of the pathological changes seen in these animals was apparently the result of secondary bacterial infection, it is belived that the demonstration of inclusion bodies is a benefit in making a specific diagnosis in necropsy material and is indicative of the primary site of injury with this virus.

Although our attempt to isolate virus from the calf at 71 hours failed, the clinical response and ensuing histological changes were indicative of viral infection. The lesions suggest that serial sections might have revealed inclusions in tissues other than reported here.

Several attempts by us and Chow [3] to demonstrate inclusions in nasal smears taken from experimentally infected animals early in the course of illness have been unsuccessful.

Since intranuclear inclusions were a constant feature of the cytopathogenicity of IBR virus in bovine kidney tissue culture but had not been previously reported in necropsy material, two conditions which might affect their demonstration were considered. These were the fixatives employed and the time during the course of disease in which necropsy specimens were examined.

In the investigation of the effects of fixation, the tissue cultures infected with IBR virus were fixed with several common fixatives. A number of cultures exhibiting cytopathogenic changes induced by the virus were fixed in Bouin's fluid, Zenker's acetic, 10 per cent formalin, methyl alcohol, and acetone. All cultures were stained with hematoxylin and eosin and examined for inclusions. Of the fixatives studied, Bouin's fluid and Zenker's acetic were superior for the demonstration of inclusions. In the tissue culture preparations fixed in methyl

alcohol or acetone, nuclear detail was good but well-formed inclusions were not demonstrable. The formalin-fixed tissue culture preparations were the least desirable for studying the cytopathology. In these, inclusions bodies were not observed.

Necropsy specimens from the same calf infected with IBR were fixed in Bouin's fluid, Zenker's solution, and 10 per cent formalin. Histological preparations of these tissues were stained with hematoxylin and eosin. Well-formed inclusions were readily demonstrated in tissues fixed with Bouin's fluid and Zenker's solution. Although nuclear changes were evident, distinct inclusions were not apparent in the formalin-fixed material. These results indicate that formalin fixation is not as effective as the acid fixative (Bouin's or Zenker's solution) in demonstrating these inclusions.

SUMMARY

A description was given of intranuclear inclusion bodies occurring in epithelial cells of the respiratory tract of calves inoculated with three strains of infectious bovine rhinotracheitis. The inclusions occurred as early as 36 hours postinfection and persisted through 60 hours.

Acid fixations with Zenker's and Bouin's fluid were superior to formalin in the demonstration of well-formed inclusions.

The value of inclusions as an aid in clinical diagnosis is questionable in view of their transitory nature.

References

[1] Cheatham, W. J., and Crandell, R. A.: Occurrence of Intranuclear Inclusions in Tissue Cultures Infected with Virus Infectious Bovine Rhinotracheitis. Proc. Soc. Exptl. Biol. & Med., *96*, (1957): 536–538.

[2] Chow, T. L., Fort Collins, Colo.: Personal communication, 1958.

[3] Jensen, R., Griner, L. A., Chow, T. L., and Brown, W. W.: Infectious Rhinotracheitis in Feedlot Cattle. I. Pathology and Symptoms. Proc. U.S. Livestock San. A (1955): 189–199.

[4] Madin, S. H., Andrise, P. C., and Darby, N.: The *in Vitro* Cultivation of Tissues of Domestic and Laboratory Animals. Am. J. Vet. Res., *18*, (Oct., 1957): 932–941.

[5] Madin, S. H., York, C. J., and McKercher, D. G.: Isolation of the Infectious Bovine Rhinotracheitis Virus. Science, *124*, (1956): 721–722.

[6] McKercher, D. G., Moulton, J. E., and Jasper, D. E.: Virus and Virus-like Disease Entities New to California. Proc. U.S. Livestock San. A. (1954): 260–269.

[7] McKercher, D. G., Moulton, J. E., Kendrick, J. W., and Saito, J. P.: Recent Developments on Upper Respiratory Diseases of Cattle. Proc. U.S. Livestock San. A. (1955): 151–167.

[8] McKercher, D. G., Moulton, J .E., Madin, S. H., and Kendrick, J. W.: Infectious Bovine Rhinotracheitis—A Newly Recognized Virus Disease of Cattle. Am. J. Vet. Res., *18*, (April, 1957): 246–256.

[9] Miller, N. J.: Infectious Necrotic Rhinotracheitis of Cattle. J.A.V.M.A., *126*, (June, 1955): 463–467.

[10] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493–497.

[11] Schroeder, R. J., and Moys, M. D.: An Acute Upper Respiratory Infection of Dairy Cattle. J.A.V.M.A., *125*, (Dec., 1954): 471–472.

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Thermal Inactivation and Antigenicity Studies of Heated Tissue Suspensions Containing Foot-and-Mouth Disease Virus

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THE HIGHLY CONTAGIOUS NATURE of foot-and-mouth disease (FMD) makes it imperative that strict safety precautions be observed to insure control of the virus. The use of noninfectious antigens for serological tests is desirable so that laboratory procedures, such as the complement-fixation (CF) test, may be performed without exposing susceptible animals or contaminating laboratory work areas.

In these studies, attempts were made to prepare a noninfectious FMD viral antigen for use in the CF test. Infected tissue suspensions were heated in a water bath at various temperatures for different periods of time followed by determinations of antigenicity and infectivity of these materials. Such studies led to determination of the thermal death point of the virus.

REVIEW OF LITERATURE

A survey of the literature showed that details were lacking on the effects of heat on the infectivity of materials containing foot-and-mouth disease virus (FMDV). Data concerning test animals, routes of inoculation, virus source and history, volume of inoculum, pH of the suspending medium, methods of heating, and other pertinent details were omitted in many of the previous reports.

Initial work on the effect of heat on FMDV was done by Loeffler and Frosch [17, 18] during the latter part of the nineteenth century. These investigators observed that FMDV contained in vesicle fluid from lesions on the bovine tongue was inactivated by heating at 31 C. for 24 hours, at 37 C. for 12 hours, and at 70 C. for one half hour. Virus infectivity was not always destroyed when it was heated at 60 C. for one half hour, as determined by infectivity tests in cattle and swine. Data concerning doses, routes of inoculation, and dilutions were not given.

Hecker [13] reported that FMDV contained in vesicular fluid, saliva, and manure was rendered inactive by heating at both 53 and 70 C. for as little as three days. This was demonstrated by the absence of lesions in cattle after intravenous inoculation and application of such material to scarified oral mucosae.

British workers [9, 10] studied the effect of heat on guinea pig vesicular fluid and found that a temperature of 55 C. inactivated the infectious property of the virus in 15 to 40 minutes when suspended in saline solution, and in one or two minutes when suspended in phosphate solution at a pH of 7.6. Olitsky *et al.*[20] reported that the infectious property of FMDV contained in guinea pig vesicular fluid survived for three but not five days when diluted in phosphate buffer at a pH of 7.6 and held at 37 C. Sichert-Modrow [24] reported that the infectivity present in dried guinea pig vesicular fluid survived for two and one half hours when heated at 70 C., and for three minutes at 122 C.

It was stated in the first progress report of the British Foot-and-Mouth Disease Research Committee [5] that infectivity of FMDV was destroyed at 37 C. in 24 hours when diluted in phosphate-buffered glycerol at a pH of 7.6, but survived for five days at 37 C. when diluted in phosphate buffer at a pH of 7.6.

Trautwein [27] reported that FMDV in vesicle coverings from guinea pigs and swine was not infectious for guinea pigs when suspended in saline solution and heated at 60 C. for five minutes. In suspensions of bovine tongue epithelium, virus infectivity survived for six days when heated at 37 C.[30] When sweetbreads from infected animals were heated in water for as long as 22 minutes at 60 C. and suspensions were prepared from these tissues for the determination of infectivity, it was found that the virus was noninfectious.[12]

Matte and Sanz [19] found infectious defibrinated bovine blood to be noninfectious for cattle when heated at 58 and 60 C. for 15 minutes.

Virus in infected guinea pig blood survived treatment at 37.5 C. for 24 hours and at 42 C. for 12 hours.[16] Similarly, infected guinea pig blood heated at 50 C. for 150 minutes was found to be infectious, whereas when it was heated at 55 C. for 20 minutes and at 60 C. for two minutes, the infectivity was destroyed.[9] When blood was heated at 37 C., the infectious nature of FMDV was found to be active

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after 24 but not 48 hours, as shown by guinea pig infectivity tests.[20] In additional studies, the British [10] showed that a temperature of 60 C. for two minutes inactivated the virus in infectious guinea pig blood, as demonstrated by the absence of lesions in guinea pigs.

The effect of heat on the complement-fixing activity of FMDV suspensions has been given little attention. Exposure temperatures of 56 and 60 C. for as long as one hour did not significantly affect the complement-fixing activity of antigen, although infectivity was not always destroyed.[2, 6]

Reviews of certain aspects of inactivation of FMDV by heating, reported prior to 1939, occur in two monographs.[28, 29]

MATERIALS AND METHODS

Preliminary Experiments.—Several methods of heating infected tissue suspensions were employed: *(a)* virus suspensions were placed in cotton-plugged tubes which were immersed in a constant-temperature water bath; *(b)* water-tight, rubber-stoppered tubes fitted with a thermometer and containing virus suspensions were placed in a constant-temperature water bath; and *(c)* flame-sealed ampules containing virus were completely immersed in a constant-temperature water bath, with and without stirring the contents of the water bath.

In the preliminary trials, 10^{-1} dilutions of virus suspensions were heated at 56, 58, 60, 62, and 65 C. for 30 minutes. In most trials, serial tenfold dilutions of heated suspensions were inoculated into steers, guinea pigs, suckling mice, and tissue cultures of calf kidney epithelial cells in stationary tubes. Infectivity was demonstrated in each instance in one or more of the mediums used. In some instances, more infected sites on the bovine tongue and higher numbers of cultures of calf kidney cells showing cytopathogenic effects were observed after inoculation of the higher dilutions of virus than after inoculation of the lower dilutions. This reversal of infectivity, although not always observed, suggested an interference phenomenon such as has been reported with mixtures of active and inactive virus.[15]

Following these observations, it was decided that the techniques used for heat treatment of virus suspensions were not adequate for accurate control of temperature. Therefore, a unit which met the requirements for adequate control of temperature, agitation of tissue suspensions, and stirring of the contents of the water bath was devised.[7] In this unit, the temperature may be controlled to ±0.01 C.

Preparation of Virus Suspensions.—Epithelium was used as the virus source from bovine tongues and guinea pig foot pads infected with FMDV, type A, strain 119 (A-119), originally obtained from the Research Institute, Pirbright, Surrey, England. The tissues were ground in a mortar with alundum and diluted with tryptose phosphate broth at a pH of 7.4 to a 10 per cent concentration. Virus suspensions were centrifuged for 20 minutes at 700 x g and dispensed in 20-ml. amounts into flat-bottomed glass vials, measuring 25 by 95 mm. Exceptions were made, involving massive-dose inoculation experiments, where virus suspensions were heated in 125-ml. amounts in milk-dilution bottles. Each of the vials or bottles contained a plastic-covered magnet and was fitted with a calibrated thermometer fixed securely in a water-tight rubber stopper. Various temperatures and periods of exposure time were used. Timing of the heat treatment was begun when the desired temperature was reached in the suspension. Pre-exposure time ranged from three to 15 minutes, depending upon the volume of test material. After heat treatment, all materials were promptly chilled to approximately 4 C. Unheated control suspensions held at 4 C. were diluted and tested for infectivity and complement-fixing activity after the preparation of the heat-treated samples.

Infectivity Tests—Small-Volume Inoculums.—Heated tissue suspensions were routinely diluted from 10^{-1} to 10^{-4} in tryptose phosphate broth for infectivity tests in cattle, guinea pigs, and mice. For infectivity tests in tissue cultures, the suspensions were diluted in tissue culture fluid consisting of 2.0 per cent bovine serum and 0.5 per cent lactalbumin hydrolysate in Hanks' balanced salt solution.[1] Unheated virus suspensions were diluted in the appropriate fluids described above. The 50 per cent lethal dose ($l.d._{50}$) and 50 per cent infectious dose ($i.d._{50}$) titers obtained using these various mediums were calculated according to the method of Reed and Muench.[21]

Several experiments were designed to determine the effects of removal of extraneous tissue components which might possibly protect virus particles from heat. Infected bovine tongue epithelium was used as the virus source. In one experiment, virus was prepared in tryptose phosphate broth and filtered through a Selas 03 filter. The filtrate was heated as previously described at 70 C. for varying periods of time. In another experiment, virus was prepared in phosphate buffered saline solution at a pH of 7.4 and centrifuged at 30,000 r.p.m. for 90 minutes at 105,500 x g. The sediment was resuspended in buffered saline solution and heated as before at 70 C.

Cattle.—Grade Hereford steers, 18 to 24 months old and weighing 600 to 700 lb. each, were inoculated with 0.1-ml. amounts by the intradermolingual (i.d.l.) route in five sites for each dilution, using four dilutions per tongue and 2 steers per sample.[24] The animals were observed daily for as long as 14 days for vesiculation, elevated body temperature, and other signs of FMD. Steers not showing evidence of infection were tested for susceptibility by the inoculation of 2.0 ml. in approximately 20 sites by the i.d.l. route with a 10^{-1} suspension of active FMDV of bovine origin.

Serum samples, collected from cattle at various intervals following inoculation with control and heated materials, were tested for the presence of specific antibody. The virus-neutralization test in suckling mice [25] and the less sensitive complement-fixation test [6, 23, 26] were used.

Guinea Pigs.—Guinea pigs, weighing approxi-

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mately 600 Gm. each, were inoculated in each metatarsal pad, using 0.1-ml. amounts by the tunneling technique and 10 guinea pigs per dilution. The animals were observed for approximately 14 days for vesiculation and other signs of infection. Guinea pigs not responding to primary inoculations during the observation period were tested for susceptibility by inoculation of each pad with 0.1 ml. of a 10^{-1} suspension of active guinea pig-adapted FMDV-A119.[5]

Suckling Mice.—Mice, 7 to 9 days of age, were inoculated intraperitoneally with 0.05-ml. amounts of the heated suspensions or unheated control suspensions, using 10 mice per dilution. The mice were observed daily for paralysis and death. Final readings were made 14 days after inoculation.[25]

Tissue Culture.—Trypsin-dispersed calf kidney cells were propagated in stationary tubes for seven days in 0.8 ml. of tissue culture fluid. Each tube was inoculated with 0.4 ml. of the diluted tissue suspension, using 10 tubes of cultured tissue cells per dilution. The tissue cultures were observed for 48 hours for cytopathogenic effect produced by FMD virus.[1]

Infectivity Tests—Large-Volume Inoculums.—The possibility existed that, although steers were inoculated with small volumes of infected tissue suspensions heated at 85 C. for six hours and found to be noninfective, such might not be the case if dilutions of such preparations were inoculated in large volumes. A 10^{-1} suspension of infected bovine tongue epithelium was prepared as previously described. The supernatant fluid of the suspension was heated at 85 C. for six hours. After being heated, the material was diluted in series of 10^{-1} to 10^{-5}, inclusive.

Cattle.—In the massive inoculation experiments, 5.0 ml. of the 10^{-1} suspensions was inoculated in approximately 20 sites, 0.25 ml. per site, by the i.d.l. route into each of 2 steers. Additional steers were inoculated by the intravenous (i.v.), subcutaneous (s.c.), and intramuscular (i.m.) routes, using 50.0 ml. in each of 2 steers for each route.

Steers were inoculated by the i.m. route with 50-ml. amounts and by the i.d.l. route with 5-ml. amounts of the dilutions 10^{-1} to 10^{-5} of the specially designed experiment to test infectivity of large volumes of dilutions of heated preparations.

The original virus, in all instances, was titrated in 2 steers by the inoculation of dilutions 10^{-5} to 10^{-6} via the i.d.l. route. The inoculated cattle were held for 14 days and challenged with dilutions of the original virus held as frozen tissue for that purpose.

Blood samples from cattle inoculated with heat-treated virus were taken seven and 14 days post-inoculation, and the 14-day samples were examined for complement-fixing antibodies by the Traub-Möhlmann test.[26] Portions of the same samples were tested for neutralizing antibody in a virus-neutralization test in suckling mice.

Guinea Pigs.—Guinea pigs were inoculated in the rear plantar pads with dilutions of original virus containing antibiotics (1,000 units penicillin and 1 mg. streptomycin per ml. of suspension), using 5 animals per dilutions of 10^{-3} to 10^{-6}. Heated virus suspensions were also tested in guinea pigs in dilutions of 10^{-1} to 10^{-3}. Each guinea pig received 5 ml. intramuscularly, with 5 or 10 animals per dilution. All guinea pigs were challenged with active virus by pad inoculation after 14 days' observation.

Tissue Culture.—Cultures of bovine kidney cells were inoculated with dilutions of 10^{-4} to 10^{-8} of original virus to which antibiotics were added. Ten tubes of cultured cells were used per dilution. In the same manner, dilutions of 10^{-1} to 10^{-3} of the heated virus suspension were inoculated into cultured kidney cells.

Laboratory Safety Tests.—In a determination of the thermal death point of a relatively heat-resistant infectious agent such as FMDV, when animals are the test mediums experimental conditions must be critically controlled to insure that virus is not transferred from infected to clean areas or animals.

In one of three experiments, the infectivity was not destroyed in virus heated at 85 C. for four hours. The virus used in this one trial was found to have an $i.d._{50}$ of 9.6, which is at least 1.5 log units higher than the virus used in the other two trials.

When the suspensions of infected bovine tongue epithelium were heat-treated at 80 C. for six hours, infectivity was also present. Because of the wide differences in the findings reported here and those appearing in the literature,[25, 29] it was necessary to determine that virus was not accidentally transferred to the animals given heat-treated virus. For this series of tests, virus heated at 80 C. for six hours was used.

In these trials, routine laboratory operating procedures were followed as in all preceding experiments. Experimental animal-holding rooms were decontaminated and stocked with steers for a two-week observation period prior to inoculation of heated materials. The animals were observed daily for signs of infection and elevation of body temperature to test for residual infectious virus in the animal rooms. At the end of the two-week holding period, 1 of 2 control steers in each isolation room was inoculated by the i.d.l. route in multiple sites, using 5-ml. amounts of a 10^{-1} suspension of normal bovine tongue epithelium heated at 85 C. for six hours. The other normal control steer in the room was not inoculated but served as a contact control for the inoculated animal in the same room. Other steers were then inoculated with suspensions of infected bovine tongue epithelium, heated at 85 C. for six hours and known to be noninfectious on the basis of data developed in past trials. A 10^{-1} dilution was inoculated into each of 2 steers per route, using 50-ml. amounts by the i.v., s.c., and i.m. routes and 5 ml. by the i.d.l. route. Additional steers were then inoculated in the same manner as described above with material heated at 80 C. for six hours which, on the basis of previous trials, was thought to be infectious.

The animals were in suites composed of two rooms separated by a shower. Each of the animal rooms was separated from the shower by an air-tight door. Steers inoculated with a heated suspension of normal, noninfected, bovine tongue epithelium were located in one room, while steers inoculated with heated, infected suspensions were in the other. All steers were held for two weeks under these conditions. At the end of this period, all animals inoculated with heated suspensions and not showing infection were challenged and held for an additional two weeks to determine possible spread of active virus to control animals inoculated with normal heated samples in the adjoining room of each suite.

The unheated virus control suspension and the normal tissue suspensions heated at 80 and 85 C. for six hours were also inoculated into tissue cultures of calf kidney epithelial cells, using the stationary tube and plaque assay techniques. Ten tubes and three monolayer cultures were used for each dilution of 10^{-1} through 10^{-6}.

Mice, 7 to 9 days old, were inoculated by the intraperitoneal route in 0.05-ml. amounts with dilutions of the unheated virus control suspension and the two heated infected suspensions, using 10 mice per dilution, 10^{-1} through 10^{-6}. In addition, 100 mice were similarly inoculated in 0.1-ml. amounts with a 10^{-1} suspension for each of the two heated infected suspensions.

Guinea pigs were inoculated in the foot pad with 0.1-ml. dilutions (10^{-1} through 10^{-5}) of the unheated virus control suspension and the two heated infected suspensions, using 5 guinea pigs per dilution. In addition, guinea pigs were inoculated by the i.m. route with 5-ml. amounts, using 10 guinea pigs for each of the two heated suspensions.

All mice and guinea pigs were held for two weeks and, in the absence of lesions, the guinea pigs were challenged with active bovine-adapted FMDV.

Complement-Fixation Tests.—Heated and control suspensions of FMD virus were prepared in two-fold serial dilutions in 0.25 ml. of physiological saline solution per tube. Anti-FMD-A119 guinea pig serum which had been heated at 56 C. for 30 minutes was added in 0.25-ml. amounts to each tube. Normal guinea pig serum containing 1.6 units of complement per 0.25 ml. was added, and the mixtures were shaken and placed in a water bath at 37 C. for 20 minutes. After incubation, the hemolytic system was added. This was composed of four units of amboceptor in 0.25 ml. and 0.25 ml. of a sheep erythrocyte suspension which had a hemolyzed optical density of 0.50 ± 0.01 when measured at 580 mμ in a spectrophotometer. The mixtures were shaken and returned to the water bath at 37 C. for 30 minutes, after which interval the presence or absence of hemolysis was determined. No hemolysis was designated as 4 (positive), increasing degrees of hemolysis as 3, 2, and 1, and complete hemolysis as 0 (negative). Controls were included in all tests for antigen, serum, complement, and hemolytic system.[25]

Virus-Neutralization Test.—Qualitative neutralization tests were made on serums obtained from cattle inoculated with heated materials by inoculating suckling mice with dilutions of serum with a constant dose of virus. Negative and positive serum controls were included in all test.[25]

RESULTS

Infectivity Results.—Representative infectivity results on the effect of heating bovine tongue epithelial suspensions containing FMD virus at various temperatures and periods of time are given (table 1). Representative data are given for 2 steers in all tables.

In all instances, as evidenced by the appearance of vesicles on tongue epithelium of test steers and the development of cytopathogenic changes in tissue cultures of calf kidney cells, all virus suspensions heated at 56 C. were determined to be infectious. The suckling mouse and guinea pig did not show evidence of infection in any of these tests.

All samples heated at 70 C. for as long as six hours and inoculated into the tongue epithelium of steers were shown to contain infectious virus, whereas in tissue cultures, virus infectivity was demonstrated only in the materials heated at 70 C. for 30 minutes. Those samples heated for longer periods did not produce changes in tissue cultures which were characteristic of FMD infection.[1]

Reversal of virus infectivity was observed only in the bovine tongue and tissue culture infectivity series. In approximately 30 per cent of the studies, there were more infected sites in the tongue and tissue cultures in the higher dilutions than in the lower dilutions. In many observations, the 10^{-1} dilution was devoid of demonstrable infectivity. It was observed that as the temperature for inactivation of the virus suspensions was increased, the degree of reversal of infectivity was diminished.

Further attempts to inactivate FMDV were made by increasing the temperature to 80 C. with exposure times of 30 minutes, two, four, and six hours. Results of these experiments using bovine and guinea pig virus are given (tables 1 and 2). Bovine FMDV suspensions treated in this manner were infectious only in cattle and were evidenced by local and generalized lesions on the tongue and generalized foot lesions in animals inoculated with material exposed to 80 C. for periods extending to four hours. Those samples heated for six hours at 80 C.

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TABLE 1—The Effect of Heat on the Infectivity of Bovine Tongue Epithelium Containing Foot-and-Mouth Disease Virus, Using Small Volumes of Inoculum in Cattle and Tissue Cultures

Exp. No.	Titers* of virus suspensions: Bovine tongue†	Titers* of virus suspensions: Tissue culture†	Temperature	Duration of heat exposure	Dilutions of heated virus suspensions inoculated	Bovine tongue (data on 2 steers)		Tissue culture
					10^{-1}	0/5**	0/5**	0/10**
				30 min.	10^{-2}	0/5	0/5	0/10
					10^{-3}	2/5	0/5	1/10
					10^{-4}	3/5	1/5	3/10
					10^{-1}	5/5	0/5	0/10
8	8.8	7.2	56 C.	45 min.	10^{-2}	5/5	3/5	3/10
					10^{-3}	5/5	0/5	2/10
					10^{-4}	2/5	0/5	6/10
					10^{-1}	1/5	0/5	0/10
				1 hour	10^{-2}	0/5	1/5	0/10
					10^{-3}	2/5	3/5	0/10
					10^{-4}	3/5	0/5	2/10
					10^{-1}	0/5	0/5	1/10
				2 hours	10^{-2}	0/5	2/5	4/10
					10^{-3}	1/5	0/5	2/10
13	7.7	6.9	56 C.		10^{-4}	0/5	0/5	5/10
					10^{-1}	2/5	2/5	0/10
				4 hours	10^{-2}	0/5	1/5	3/10
					10^{-3}	0/5	0/5	6/10
					10^{-4}	0/5	0/5	3/10
					10^{-1}	3/5	2/5	0/10
				6 hours	10^{-2}	2/5	0/5	3/10
					10^{-3}	1/5	0/5	2/10
					10^{-4}	0/5	0/5	0/10
					10^{-1}	2/5	5/5	2/10
				12 hours	10^{-2}	0/5	5/5	2/10
					10^{-3}	0/5	1/5	4/10
14	6.9	7.2	56 C.		10^{-4}	0/5	0/5	0/10
					10^{-1}	1/5	2/5	0/10
				18 hours	10^{-2}	2/5	0/5	1/10
					10^{-3}	0/5	0/5	3/10
					10^{-4}	0/5	0/5	0/10
					10^{-1}	3/5	1/5	0/10
				24 hours	10^{-2}	4/5	0/5	0/10
					10^{-3}	1/5	0/5	1/10
					10^{-4}	4/5	1/5	0/10
					10^{-1}	2/5	Generalized	0/10
9	8.5	7.1	70 C.	30 min.	10^{-2}	0/5	lesions on	2/10
					10^{-3}	0/5	tongue and	2/10
					10^{-4}	4/5	feet	4/10
					10^{-1}	2/5		0/10
				1 hour	10^{-2}	0/5	Steer	0/10
					10^{-3}	0/5	died	0/10
12	8.3	6.1	70 C.		10^{-4}	0/5		0/10
					10^{-1}	3/5	2/5	0/10
				1.5 hours	10^{-2}	0/5	1/5	0/10
					10^{-3}	0/5	0/5	0/10
					10^{-4}	0/5	0/5	0/10
					10^{-1}	5/5	0/5	0/10
				2 hours	10^{-2}	5/5	2/5	0/10
					10^{-3}	4/5	4/5	0/10
					10^{-4}	1/5	2/5	0/10
					10^{-1}	5/5	1/5	0/10
15	7.5	6.4	70 C.	4 hours	10^{-2}	5/5	0/5	0/10
					10^{-3}	1/5	0/5	0/10
					10^{-4}	2/5	0/5	0/10
					10^{-1}	2/5	Generalized	0/10
				6 hours	10^{-2}	0/5	lesions on	0/10
					10^{-3}	0/5	tongue and	0/10
					10^{-4}	0/5	feet	0/10

(Table 1 continued on next page)

TABLE 1 (Continued)—The Effect of Heat on the Infectivity of Bovine Tongue Epithelium Containing Foot-and-Mouth Disease Virus, Using Small Volumes of Inoculum in Cattle and Tissue Cultures

Exp. No.	Titers* of virus suspensions: Bovine tongue†	Titers* of virus suspensions: Tissue culture†	Temperature	Duration of heat exposure	Dilutions of heated virus suspensions inoculated	Bovine tongue (data on 2 steers)	Tissue culture
18	7.8	....	80 C.	30 min.	10^{-1}	0/5 2/5	0/10
					10^{-2}	2/5 5/5	0/10
					10^{-3}	0/5 3/5	0/10
					10^{-4}	0/5 0/5	0/10
				2 hours	10^{-1}		0/10
					10^{-2}	Generalized lesions	0/10
					10^{-3}		0/10
					10^{-4}		0/10
				4 hours	10^{-1}		0/10
					10^{-2}	Generalized lesions	0/10
					10^{-3}		0/10
					10^{-4}		0/10
				6 hours	10^{-1}	0/5 0/5	0/10
					10^{-2}	0/5 0/5	0/10
					10^{-3}	0/5 0/5	0/10
					10^{-4}	0/5 0/5	0/10

* Average obtained in 2 steers. † 0.1 ml. per site given intradermolingually in five sites, and 0.4 ml. per tube (i.d.$_{50}$ titers corrected to 1 ml.). ** No. of sites or cultures infected/No. of sites or cultures inoculated.

did not produce evidence of infection in any medium tested with small volumes of inoculum. Animals used in these experiments were challenged and were shown to be fully susceptible to FMD virus.

The reversal of infectivity observed in previous tests was also seen in steers inoculated with material heated at 80 C. for 30 minutes, but not with the regularity observed in the materials heated at lower temperatures for shorter periods.

Slightly different results were obtained when guinea pig-pad suspensions of FMD virus were treated in essentially the same manner as in the experiments involving bovine tongue virus. Infectivity of guinea pig virus for cattle was apparently destroyed with heat treatment at 80 C. for four hours. Cultures of calf kidney cells showed cytopathogenic effect when inoculated with guinea pig virus heated at 80 C. for 30 minutes, but not after longer periods of heating. A reversal of infectivity was also observed in steers inoculated with suspensions of guinea pig-pad virus heat-treated at 80 C. for 30 minutes. Mice and guinea pigs did not show evidence of infection when inoculated with any of the heated virus suspensions.

From the results obtained in the experiments using small-volume inoculums in steers and other mediums, it was assumed that FMDV infectivity was destroyed when suspensions were heated at 80 C. for six hours. However, when massive doses of heated suspensions were inoculated into cattle by the i.v., s.c., i.m., and i.d.l. routes, infectivity was demonstrated. In two experiments (29 and 31, table 3) using inoculations of 50-ml. quantities of virus in each steer by the i.v., s.c., or i.m. routes or of a 5-ml. quantity by the i.d.l. route, infectivity was still present in virus suspensions heated at 80 C. for six hours. In experiment 29, 2 steers inoculated with 50 ml. each by the s.c. route became infected and showed generalized lesions in 13 days. Mice and guinea pigs did not show evidence of infection. In experiment 31, which was a repetition of experiment 29 using massive-dose inoculations, both steers inoculated by the i.d.l. route showed a generalized infection in eight days. One animal inoculated by the i.m. route showed a generalized infection in 12 days. Steers not responding to primary inoculations were challenged and were shown to be fully susceptible to infection.

Further trials to inactivate FMDV contained in suspensions of infected bovine tongue epithelium were attempted by extending the heat treatment to 85 C. for six hours. Massive doses of material heated in this manner were tested in cattle, suckling mice, and bovine kidney cell tissue cultures. Results of experiment 32 in cattle are summarized (table 3). In these trials, infectivity was not demonstrated in suspensions heated at 85 C. for six hours and tested in cattle, mice, and tissue cultures.

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From the results of data developed thus far, it was concluded that FMD-A119 contained in suspensions of infected bovine tongue epithelium was no longer infectious after treatment with heat at 85 C. for six hours; however, it was considered advisable to attempt to determine more accurately the time of inactivation at 85 C. Experiments were designed to treat virus suspensions for four hours at 85 C. Massive-dose inoculations of heat-treated virus were made in cattle as in previously described tests. Suckling mice and tissue cultures of calf kidney cells in Roux flasks containing 75 ml. of culture medium were inoculated with 10 ml. of the heated suspension. Guinea pigs were inoculated with 5 ml. of the suspension by the i.m. route. Typical results of experiments 35 and 36 are summarized (table 3).

In three experiments involving these conditions of heating, virus infectivity was not destroyed in one experiment (No. 35), as demonstrated by infected cattle inoculated by the i.m. route. The other cattle did not show evidence of infection, nor was infection shown in the Roux flasks or tubes of tissue cultures of calf kidney cells in mice or in guinea pigs. From this series of tests, it was concluded that infectivity was not destroyed in all instances when suspensions of bovine tongue epithelium containing virus were heated at 85 C. for four hours. In those trials in this series where infectivity was demonstrated, the $i.d._{50}$ titer per milliliter in steers was found to be 9.6, at least 1.5 log units higher than in the other two trials.

In the studies designed to remove extraneous tissue by filtration and ultracentrifugation, it was found that the virus was still infectious for cattle by the i.d.l. route when heated for as long as six hours, as demonstrated by the development of local and generalized lesions. The interference phenomenon was also observed.

TABLE 2—The Effect of Heat on the Infectivity of Guinea Pig Foot Pad Epithelium Containing Foot-and-Mouth Disease Virus, Using Small Volumes of Inoculum in Cattle and Tissue Cultures

Exp. No.	Titers* of virus suspensions: Bovine tongue†	Titers* of virus suspensions: Tissue culture†	Temperature	Duration of heat exposure	Dilutions of heated virus suspensions inoculated	Bovine tongue (data on 2 steers)		Tissue culture
28	6.8	6.6	80 C.	30 min.	10^{-1}	2/5**	4/5**	2/10**
					10^{-2}	0/5	5/5	2/10
					10^{-3}	1/5	3/5	0/10
					10^{-4}	0/5	1/5	0/10
				2 hours	10^{-1}	3/5	2/5	0/10
					10^{-2}	0/5	0/5	0/10
					10^{-3}	2/5	0/5	0/10
					10^{-4}	2/5	0/5	0/10
				4 hours	10^{-1}	0/5	0/5	0/10
					10^{-2}	0/5	0/5	0/10
					10^{-3}	0/5	0/5	0/10
					10^{-4}	0/5	0/5	0/10
				6 hours	10^{-1}	0/5	0/5	0/10
					10^{-2}	0/5	0/5	0/10
					10^{-3}	0/5	0/5	0/10
					10^{-4}	0/5	0/5	0/10

* Average obtained in 2 steers. † 0.1 ml. per site given intradermolingually in five sites, and 0.4 ml. per tube ($i.d._{50}$ titers corrected to 1 ml.). ** No. of sites or cultures infected/No. of sites or cultures inoculated.

In one experiment, a FMDV suspension which had been heat-treated at 56 C. for 90 minutes and which had shown the interference phenomenon in cattle was stored as a 10^{-1} suspension at −40 C. for six months. This material again produced the same phenomenon when inoculated into cattle after storage. The control suspension, which was also stored under similar conditions, did not show a significant change in infective titer in any of the mediums tested.

In the vast majority of all experiments, the virus suspensions were prepared in tryptose phosphate broth at a pH of approximately 7.4. After heating these suspensions, the pH values were again determined, and it was found that the pH of the heated suspensions decreased by a maximum of 0.25 unit.

In later experiments, using the virus-neutralization test in suckling mice,[25] antibodies were detected in serums of cattle inoculated with heated materials but which had not shown lesions of FMD. In three instances, antibodies were demonstrated in serums of steers after inoculation with materials heated at 85 C. for four hours. Although gross lesions were absent in some of the animals, other animals in the test series showed lesions after inoculation with 50 ml. of heated suspension.

Serum samples from animals from test series where infection was not shown did not contain complement-fixing antibodies when tested by either direct or indirect methods.[22]

In the other samples from animals showing infection, antibodies were shown only by the indirect test method. Serums from cattle which had shown neutralizing antibodies but no lesions did not show antibodies by the indirect CF test.

Laboratory Safety Tests.—The results of the experiments to test the ability to contain the virus of FMD in the large-animal compartments when both active and inactivated virus were in use are shown in table 3. No infectivity for mice, tissue culture, or guinea pigs was found for either of the suspensions heated at 85 C. for six hours or 80 C. for two hours and, therefore, these data are not detailed.

Evidence of the control of the transfer of virus from room to room may be checked by the appearance of specific signs and lesions, as well as by the presence of complement-fixing and virus-neutralizing antibodies in the serums of surviving cattle. Cattle inoculated with the heat-inactivated virus suspensions produced no complement-fixing antibodies during the 14-day observation period prior to challenge. The only cattle to show virus-neutralizing antibodies were the 2 animals which developed FMD after i.d.l. inoculation with virus suspensions heated at 80 C. for two hours. From these results, it was concluded that when FMD appeared in the inoculated animals, or serums from such animals were found to contain FMD antibodies, the infection was probably due to viable virus contained in

TABLE 3—The Effect of Heat on the Infectivity of Bovine Tongue Epithelium Containing Foot-and-Mouth Disease Virus, Using Large Volumes of Inoculum in Cattle

Exp No.	Titer* of virus suspensions	Temperature	Duration of heat exposure	Volume of 10^{-1} inoculum (ml.)	Route of inoculation	Results in 2 steers	
41	8.4	80 C.	2 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	NVL	NVL
				50	Intramuscular	NVL	NVL
				5	Intradermolingual	Gen.	Gen.
29	8.1	80 C.	6 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	Gen.	Gen.
				50	Intramuscular	NVL	NVL
				5	Intradermolingual	NVL	NVL
31	8.4	80 C.	6 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	NVL	NVL
				50	Intramuscular	Gen.	NVL
				5	Intradermolingual	Gen.	Gen.
35	9.6	85 C.	4 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	NVL	NVL
				50	Intramuscular	Gen.	Gen.
				5	Intradermolingual	NVL	NVL
36	8.1	85 C.	4 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	NVL	NVL
				50	Intramuscular	NVL	NVL
				5	Intradermolingual	NVL	NVL
32	7.7	85 C.	6 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	NVL	NVL
				50	Intramuscular	NVL	NVL
				5	Intradermolingual	NVL	NVL
41	8.4	85 C.	6 hours	50	Intravenous	NVL	NVL
				50	Subcutaneous	NVL	NVL
				50	Intramuscular	NVL	NVL
				5	Intradermolingual	NVL	NVL

* Average obtained in 2 steers (i.d.$_{50}$ titers corrected to 1 ml.); NVL = no visible lesions; Gen. = generalized lesions.

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TABLE 4—The Effect of Heat on Infectivity of Bovine Tongue Epithelium Containing Foot-and-Mouth Disease Virus, Using Large Volumes of Inoculum in Serial Dilutions in Cattle

Exp. No.	Titer* of virus suspensions	Temperature	Duration of heat exposure	Volume of inoculum (ml.)	Dilutions of heated virus suspensions inoculated	Route of inoculation	Results in each of 2 steers per dilution
40	8.1	85 C.	6 hours	50	10^{-1} to 10^{-8}	Intramuscular	NVL
				5	10^{-1} to 10^{-8}	Intradermolingual	NVL

* Average obtained in 2 steers (i.d.$_{50}$ titer corrected to 1 ml.) NVL = no visible lesions.

the inoculum, since the same operating procedures were used throughout the test. In addition, no steer inoculated with virus suspensions heated at 85 C. for six hours showed signs of FMD.

Sample data from experiments where steers were inoculated with large volumes of dilutions of viral suspensions inactivated at 85 C. for six hours are tabulated (table 4). None of the steers inoculated with dilutions of the heat-treated viral suspensions showed any signs of FMD. The guinea pigs and tissue culture similarly exhibited no signs of infectivity when inoculated with the heat-treated viral preparations. No neutralizing or complement-fixing antibodies were found in the serums of the surviving cattle after the 14-day observation period. It must be assumed on the basis of several experiments of this type that virus contained in suspensions of infected bovine tongue epithelium did not survive heat treatment at 85 C. for six hours.

TABLE 5—Complement-Fixing Activity of Bovine Tongue Epithelial Suspensions Containing Foot-and-Mouth Disease Virus Heated at Varying Periods of Time

Temperature and duration of heat exposure	Complement-fixing activity*						
	Antigen dilutions (10^{-1} stock)						Antigen controls
	Undil.	½	¼	⅛	1/16	1/32	
Unheated control	4	4	4	2	0	0	0
56 C., 24 hours	4	4	4	3	0	0	0
Unheated control	4	4	4	2	0	0	0
70 C., 30 min.	3	0	0	0	0	0	0
70 C., 2 hours	0	0	0	0	0	0	0
Unheated control	4	4	2	0	0	0	0
80 C., 30 min	0	0	0	0	0	0	0
Unheated control	4	4	4	2	0	0	0
85 C., 4 hours	0	0	0	0	0	0	0

* Against hyperimmune guinea pig serum. 4 = no hemolysis (positive); 3 and 2 = increasing degrees of hemolysis; 0 = complete hemolylis (negative).

Complement-Fixation Tests.—Representative results of tests on the complement-fixing activity of heated tissue suspensions containing FMDV against guinea pig hyperimmune serum are shown in (table 5).

Complement-fixing activity was not affected when suspensions of FMDV were heated at 56 C. for as long as 24 hours, whereas heating at 70 C. for 30 minutes diminished antigenicity significantly. All activity was destroyed when the virus suspensions were heated at 70 C. for two or more hours.

Recently, attention has been focused on the antigenicity of heated polio virus complement-fixing antigen,[23] where it was found that heating one type of virus would uncover antigens common to the three virus types. Similar CF tests were made in the studies reported here on FMDV suspensions heated at 56 and 80 C. for varying periods of time. In no instances was crossing encountered with anti-FMD, types O and C hyperimmune guinea pig serum.

Tongue epithelium, harvested from steers which had been inoculated with heated virus suspensions and which had shown lesions, was prepared for use as antigen in the CF test by grinding in a mortar and diluting with tryptose phosphate broth to a 10^{-1} dilution. Representative samples were tested with types O and C hyperimmune guinea pig serum. Significant crossing between the type A FMDV in the tissues and types O and C serum was not observed.

DISCUSSION

Although the primary purpose of this problem was not achieved, that is, the preparation by heat of a satisfactory noninfectious antigen of FMD virus for use in the CF test, an important fact was established. The FMD virus infectivity was found to be extremely resistant to destruction by heat. A similar observation was reported by Bachrach *et al.*,[2] working with tissue culture virus heated at 55 and 61 C. and inoculated into tissue cultures. Extreme heat resistance was also reported by the British Foot-and-Mouth Disease Research Committee [11] working with virus contained in dried milk. The results reported in the early literature [28, 29] were not confirmed by studies presented

here, since the thermal death point of bovine tongue FMDV A119 was established at 85 C. when tissue suspensions containing the virus were heated for six hours. Infectivity was not always destroyed when the tissue suspensions were heated at 85 C. for four hours.

The virus system of FMD has been shown to be composed of at least two particles, one of which is associated with infectivity and the other with complement-fixing activity.[4] From the data obtained in these present studies, the particle associated with complement-fixing activity appeared to be less resistant to heat than the infective particle. The results obtained showed that complement-fixing activity of the virus antigen was destroyed at lower temperatures and for shorter periods of exposure than the infective property. A decrease in antigenic activity was observed when virus suspensions were heated at 70 C. for 30 minutes, with absence of activity when the suspensions were heated at 70 C. for two or more hours.

In these studies, strict control of the heating procedures was accomplished by a method previously described.[7] The assembly provided for delicate temperature regulation to $\pm$ 0.01 C., and constant agitation of the virus suspensions and the contents of the water bath. Precise temperature determinations were made by periodic reading of the calibrated thermometer inserted in the tubes containing the suspensions of virus.

The choice of the test medium influenced the ability to detect small amounts of active FMDV. In many instances in which heated virus suspensions were inoculated into various test mediums, cattle were the most sensitive, with tissue cultures of calf kidney cells, suckling mice, and guinea pigs placed in decreasing order of sensitivity in their ability to detect virus. On very few occasions the mice or guinea pigs became infected with the heated virus suspension. In cases when the mice or guinea pigs showed evidence of infection, confirmation was made by further passage and by the CF test.

In most instances, as the temperature and the time of exposure were increased, virus was not detected in tissue cultures as frequently as by inoculation of cattle.

In the tests reported,[28, 29] undiluted heat-treated viral materials were inoculated into the test mediums. In the studies herein reported, infectivity was often detected in animals inoculated with virus dilutions where it was not detected in those given undiluted material. This phenomenon was more in evidence when virus suspensions were heated at the lower temperatures. This reversal of infectivity, which is a manifestation of an interference phenomenon, has been studied in other virus-inactivation experiments.[15]

The dose and route of inoculum were shown to be of extreme importance in the determination of the thermal death point of FMDV. Even though evidence was presented for the absence of infection in cattle inoculated with virus suspensions heated at 80 C. for six hours, further and definite confirmation of virus destruction was needed. When the lack of infectivity in the steers was observed, the animals were inoculated by the i.d.l. route in 20 sites, using 0.1 ml. of inoculum per site. When the volume of inoculum of the heated virus suspensions in cattle was increased and several routes of inoculation were used, virus infectivity was still shown in material heated at 80 C. for six hours. Only steers inoculated with 50 ml. each by the s.c. and i.m. routes and with 5 ml. by the i.d.l. route became infected. Cattle inoculated by the i.v. route did not show evidence of infections and were shown to be fully susceptible to challenge. None of the test mediums showed evidence of being infected when virus suspensions were heated at 85 C. for six hours. In these instances, the cattle were inoculated by a number of routes, using massive doses as described before. Similar studies made with suspensions heated at 85 C. for four hours showed that FMDV was not always destroyed.

It is of interest that in three isolated cases antibody responses were elicited, as shown by the results of the mouse neutralization test of serums from 3 steers inoculated with material heated at 85 C. for four hours. In 1 steer, an elevated temperature was observed, whereas in the other 2 steers, abnormal body temperatures were not encountered. Gross lesions of FMD were not found in these 3 animals. When these steers were challenged by the i.d.l. route with 2 ml. of a 10^{-1} dilution of virus, all developed typical infections.

This study suggests that the data obtained in heat-inactivation studies of other viruses be reviewed, taking into consid-

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eration the following: (1) Sensitivity of test medium should be such as to detect minute quantities of active virus in treated samples. In addition, as many susceptible mediums as possible should be used to test for innocuousness of the heated materials. (2) Samples should be diluted to eliminate the possibility of reading false negatives due to the interference phenomenon which may occur. (3) Negative results may be obtained when using small volumes of inoculums which may contain minute amounts of active virus. If the volume of inoculum is increased and inoculated via various routes, more active virus may be introduced. The various methods of inoculation also increase the probability of introducing virus by the most sensitive route, thus permitting the virus to multiply and produce infection. Suspensions containing a virus of high titer may require longer periods of exposure at higher temperatures than suspensions containing virus with a lower titer.

SUMMARY

1) Studies were made on the use of heat for the preparation of a noninfectious foot-and-mouth disease virus (FMDV) antigen satisfactory for use in the complement-fixation test.

2) Tissue suspensions of infected bovine tongue epithelium and infected guinea pig foot pad epithelium were heated under strictly controlled conditions at temperatures of 56 C. through 85 C. for 30 minutes to 24 hours.

3) Dilutions of the heated suspensions were inoculated in small doses into bovine tongues, guinea pigs, cultures of calf kidney cells, and suckling mice. Undiluted suspensions were inoculated in large doses into cattle by several routes and into guinea pigs and tissue cultures.

4) The infectious property of FMDV was destroyed by heating tissue suspensions at 80 C. for six hours but not at 80 C. for four hours, as demonstrated by inoculation of small doses of inoculum (2 ml.) in cattle.

5) Large doses of inoculum (50 ml.) which were heated at 80 C. for six hours were infective, as demonstrated in cattle.

6) Infectivity of FMDV was not demonstrable when tissue suspensions were heated at 85 C. for six hours and inoculated in large doses (50 ml.) by several routes in steers. Infectivity was shown when virus was heated at 85 C. for four hours and inoculated in the same manner.

7) Heating the tissue suspensions at 56 C. for as long as 24 hours did not completely inactivate the virus or destroy the complement-fixing activity.

8) Complement-fixing activity was diminished when suspensions were heated at 70 C. for 30 minutes. A temperature of 70 C. or above for more than 30 minutes destroyed the complement-fixing activity of the virus antigen.

9) Considerations for future work and implications of heat-inactivation studies in the virus field are discussed.

References

[1] Bachrach, H. L., Hess, W. R., and Callis, J. J.: Foot-and-Mouth Disease Virus: Its Growth and Cytopathogenicity in Tissue Culture. Science, *122*, (1955): 1269–1270.

[2] Bachrach, H. L., Breese, S. S., Jr., Callis, J. J., Hess, W. R., and Patty, R. E.: Inactivation of Foot-and-Mouth Disease Virus by pH and Temperature Changes and by Formaldehyde. Proc. Soc. Exptl. Biol. & Med., *95*, (1947): 147–152.

[3] Bradish, C. J., Brooksby, J. B., and Dillon, J. F.: Biophysical Studies of the Virus System of Vesicular Stomatitis. J. Gen. Microbiol., *14*, (1956): 290–314.

[4] Bradish, C. J., Brooksby, J. B., Dillon, J. F., and Norambuena, M.: Ultracentrifugal Studies of the Infective and Complement-Fixing Components in the Virus System of Food-and-Mouth Disease. Proc. Roy. Soc. London, series B, *140*, (1952): 107–127.

[5] Brooksby, J. B.: The Antibodies in Foot-and-Mouth Disease. Agricultural Research Council Report Series No. 9, His Majesty's Stationery Office, London, 1949.

[6] Brooksby, J. B.: The Technique of Complement Fixation in Foot- and-Mouth Disease Research. Agricultural Research Council Report Series No. 12, Her Majesty's Stationery Office, London, 1952.

[7] Dimopoullos, G. T., Fellowes, O. N., Callis, J. J., Poppensiek, G. C., Tessler, J., and Hess, W. R.: Heat-Inactivated Vesicular Stomatitis Virus as Antigen in the Complement-Fixation Test. Am. J. Vet. Res., *18*, (July, 1957): 688–690.

[8] (Great Britain) Ministry of Agriculture and Fisheries: First Progress Report of the Foot-and-Mouth Disease Research Committee. His Majesty's Stationery Office, London, 1925.

[9] (Great Britain) Ministry of Agriculture and Fisheries: Second Progress Report of the Foot-and-Mouth Disease Research Committee. His Majesty's Stationery Office, London, 1927.

[10] (Great Britain) Ministry of Agriculture and Fisheries: Third Progress Report of the Foot-and-Mouth Disease Research Committee. His Majesty's Stationery Office, London, 1928.

[11] (Great Britain) Ministry of Agriculture and Fisheries: Fourth Progress Report of the Foot-and-Mouth Disease Research Committee. His Majesty's Stationery Office, London, 1931.

[12] (Great Britain) Ministry of Agriculture and Fisheries: Fifth Progress Report of the Foot-and-Mouth Disease Research Committee. His Majesty's Stationery Office, London, 1937.

[13] Hecker: Untersuchungen über die Abtödtung des Contagiums der Maul- und Klauenseuche im Dünger und in Tiefställen. Berl. tierärtzl. Wchnschr., *1*, (1899): 6–7.

[14] Henderson, W. M.: The Quantitative Study of Foot-and-Mouth Disease Virus. Agricultural Research Council Report Series No. 8, His Majesty's Stationery Office, London, 1949.

[15] Henle, W.: Interference Phenomena Between Animal Viruses: A Review. J. Immunol., *64*, (1950): 203–236.

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[16] Klobouk, A.: Über Einfluss verschiedener Wärmegrade auf das Virus der Maul- und Klauenseuche. Das Verhalten des Virus im Froschkörper. Zentralbl Bakt., *81*, (1926): 111.

[17] Loeffler, F., and Frosch, P.: Summarischer Bericht über die Ergebnisse der Untersuchungen der Kommission zur Erforschung der Maul- und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. Zentralbl. Bakt., *22*, (1897): 257–259.

[18] Loeffler, F., and Frosch, P.: Berichte der Kommission zur Erforschung der Maul- und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. Zentralbl. Bakt., *23*, (1898): 371–391; also in Deutsche Med. Wchnschr., *24*, (1898): 80–83, 97–100.

[19] Matte, E., and Sanz, B.: Quelques essais de vaccination preventive contre la fièvre aphteuse. Bull. Soc. path. Exot., *14*, (1921): 523–529.

[20] Olitsky, P. K., Traum, J., and Schoening, H. W.: Report of the Foot-and-Mouth Disease Commission of the United States Department of Agriculture, U.S.D.A. Tech. Bull. 76, 1928.

[21] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent End Points. Am. J. Hyg., *27*, (1938): 493–497.

[22] Rice, C. E., and Brooksby, J. B.: Studies of the Complement-Fixation Reaction in Virus Systems. V. In Foot-and-Mouth Disease Using Direct and Indirect Methods. J. Immunol., *71*, (1953): 300–310.

[23] Schmidt, N. J., and Lennette, E. H.: Modification of the Homotypic Specificity of Poliomyelitis Complement-Fixing Antigens by Heat. J. Expti. Med., *104*, (1956): 99–120.

[24] Sichert-Modrow, I.: Haltbarkeit des Maul- und Klauenseucheerregers bei verschiedenen Temperaturen. Zentralbl. Bakt., *119*, (1930): 17–19.

[25] Skinner, H. H., Henderson, W. M., and Brooksby, J. B.: Use of Unweaned White Mice in Foot-and-Mouth Disease Research. Nature, *169*, (1952): 794–797.

[26] Traub, E., and Möhlmann, H.: Typbestimmung bei Maul- und Klauenseuche mit Hilfe der Komplementbindungsprobe. II. Versuche mit Meerschweinchenserum und Rinderantigen. Zentralbl. Bakt., *150*, (1943): 300–310.

[27] Trautwein, K.: Versuche zur Tenazität des Maul- und Klauenseuchevirus in der Aussenwelt. Arch. wissensch. u. prakt. Thierh., *54*, (1926): 273–279.

[28] Trautwein, K.: Maul- und Klauenseuche. Ergebn. Hyg. Bakt., *10*, (1929): 585–588.

[29] Waldmann, O., and Nagel, H. C.: Die Maul- und Klauenseuche. In "Handbuch der Virus-Krankheiten," E. Gildermeister, E. Haagen, and O. Waldmann, ed. Vol. I, Gustav Fischer, Jena, (1939).

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Immunological Studies on *Dictyocaulus Viviparus* Infection in Calves —Double Vaccination with Irradiated Larvae

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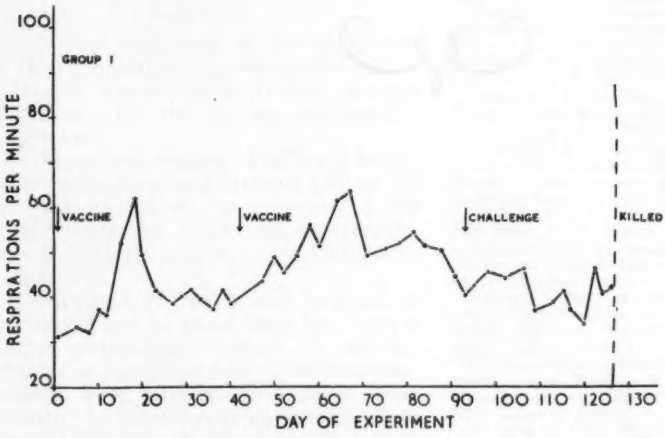
WE HAVE previously shown that it is possible artificially to immunize cattle against parasitic bronchitis due to *Dictyocaulus viviparus* infection by administering to them irradiated third-stage larvae.[1-3, 6] In laboratory experiments, a high degree of immunity was produced using 4,000 partially inactivated larvae, but this dosage level gave rise to mild pulmonary complications;[6] in the field trails,[1, 3] the immunizing dose was reduced to 1,000 larvae. This latter dose did not give rise to undesirable pulmonary signs and gave an acceptable degree of immunity. It did not, however, completely prevent the disease when the vaccinated animals were exposed to high challenge levels of parasites.[1]

The present experiment was designed to test the effect of administering two doses of irradiated larvae before exposing the animals to a high challenge infection.

MATERIALS AND METHODS

Fifty Ayrshire calves were bought when 3 days old and were raised parasite free in individual isolation stalls.

Third-stage infective larvae of *D. viviparus* were cultured [2] and subjected to a dose of 40,000 roentgens from an x-ray source as described previously.[6]



Graph 1—Mean respiratory rates, group 1.

The methods of administering larvae to the calves, estimating fecal larval output, counting the number of worms in the bronchi, and examining the calves for clinical and pathological changes were the same as described in previous reports.[2, 4, 5]

The 50 calves were allotted to five groups of 10. At the commencement of the experiment (day 0), each calf in group 1 was given 1,000 irradiated larvae. Each calf was given a further dose of 4,000 irradiated larvae 42 days later. On day 93, each calf was challenged with 10,000 normal, infective, third-stage larvae. The experiment was terminated by killing the calves on day 126. At this time, fecal larval examinations were made, the number of

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TABLE 1—Parasitological and Pathological Results of the Challenge Infection

Group	No. of larvae			Mean larvae/Gm. of feces	Mean No. of adult worms at necropsy	Mean lesion score
	1st vaccine	2nd vaccine	Challenge			
1	1,000	4,000	10,000	0	0	0.1
2	1,000	2,000	10,000	0	0	0.1
3	1,000	1,000	10,000	0	0	0.2
4	1,000	.....	10,000	285	820	7.3
5 (A)	.....	.....	10,000	454	897	7.2
(B)	.....	.....	5,000	300	1,020	7.2

worms in each pair of lungs was counted, and an assessment of the degree of pulmonary consolidation was made using the lesion score method.[4-6] Histological examination was made of affected lung tissue.

The calves of groups 2 and 3 were treated similarly except that their second vaccinating doses were 2,000 and 1,000 irradiated larvae, respectively.

The calves of group 4 were given only the initial irradiated 1,000 larvae at day 0 and were challenged with 10,000 normal larvae as before on day 93.

Group 5 was divided into two subgroups, A and B; each of the 5 calves of group A were given 10,000 viable larvae on day 93 and the 5 calves of group B were given 5,000 normal larvae. Animals in the subgroup which were infected with 5,000 normal larvae were included in case a heavy mortality should result among the 10,000 challenge group during the prepatent period; in the latter case, a quantitative assessment of response to challenge would have been impossible, as an accurate worm count could not have been obtained.

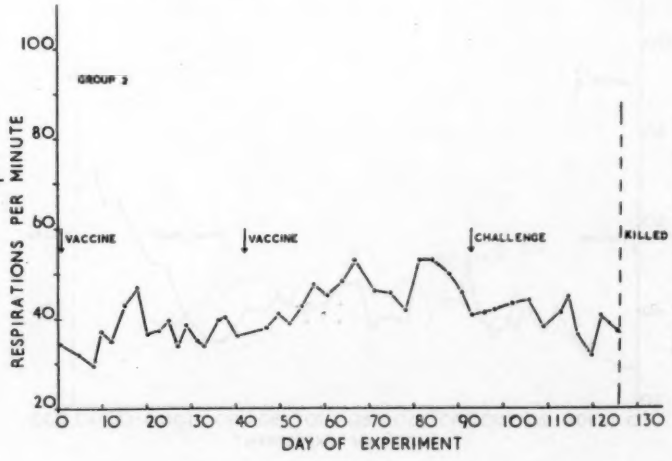
During the course of the experiment, a clinical examination was carried out on each calf twice weekly; the respiratory rate and the degree of coughing was noted. Each calf was weighed once weekly from two weeks before challenge until the end of the experiment.

In addition to these calves, two other groups (6 and 7) were used to control the viability of the larvae used in making the two batches of vaccine and to give comparisons between group respiratory rates. Each calf in group 6 was given 4,000 normal infective larvae at day 0 and the calves of group 7 were given 4,000 normal larvae on day 42.

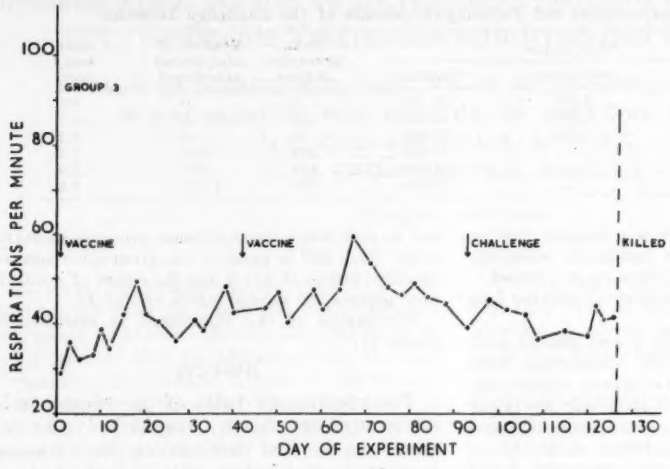
The design of the experiment is summarized (table 1).

RESULTS

The respiratory rates of the corresponding groups are shown (graphs 1–4). In response to the first vaccination, there was an increase in respiratory rate in each of these groups which reached its maximum about the eighteenth day. There was no significant statistical difference in response between these groups. This transient rise in respiratory rate has been noticed in almost all calves vaccinated by this method and is due to a few irradiated larvae reaching the lungs and producing the quickly resolving alveolar and bronchial lesions which we have described previously.[6] The respiratory rate quickly fell away to normal by about the twenty-fifth day whereas, in the control group (group 6), the respiratory rate continued to rise, reaching a maximum between the thirtieth and fortieth day, when a fully patent infection had developed.



Graph 2—Mean respiratory rates, group 2.

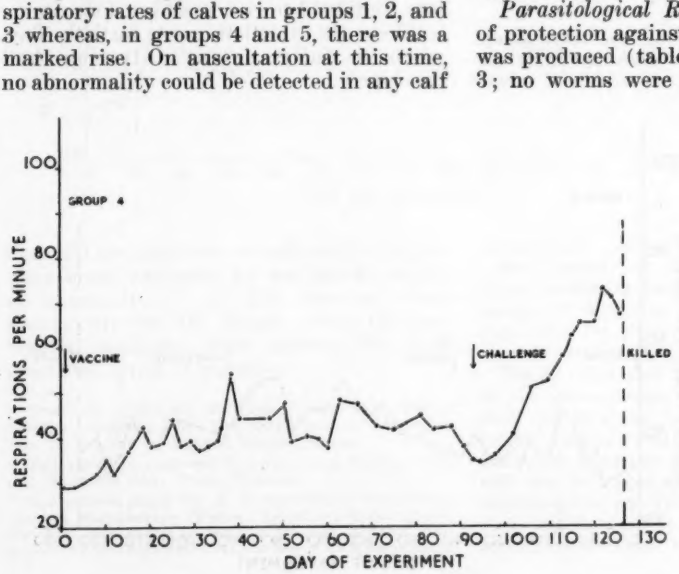


Graph 3—Mean respiratory rates, group 3.

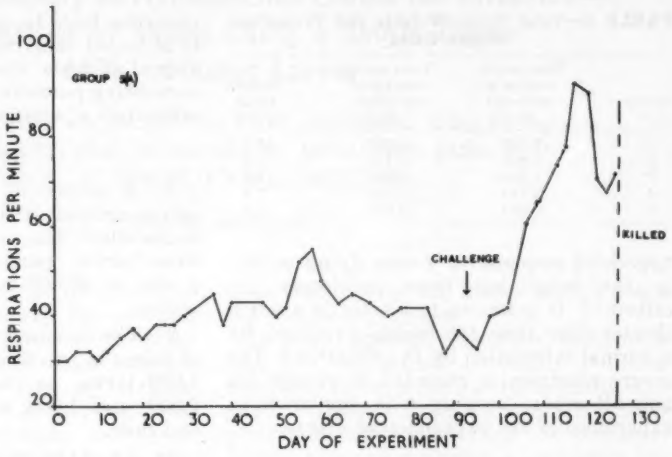
After the second vaccinating dose in groups 1, 2, and 3, there was an apparent rise in respiratory rate reaching a peak between 60 and 70 days, but this is not significantly different from the respiratory rates of calves in group 5 (graph 5), which reached 50 per minute at that time during a spell of warm weather. The calves of this latter group (destined to be the challenge control) had not at that time been interfered with experimentally in any way. After challenge with 10,000 larvae on the ninety-third day, there was no rise in respiratory rates of calves in groups 1, 2, and 3 whereas, in groups 4 and 5, there was a marked rise. On auscultation at this time, no abnormality could be detected in any calf in the first three groups; marked clinical respiratory abnormalities were present in calves in groups 4 and 5. The calves of group 5A were considered to be moribund shortly before the termination of the experiment.

The total and percentage weight gains of the groups are shown (table 2). The calves in the double vaccination groups gained approximately 18.0 per cent in weight during this time, those in the single vaccination group gained 7.6 per cent, and the challenge controls gained only 5.0.

Parasitological Results.—A high degree of protection against the challenge infection was produced (table 1) in groups 1, 2, and 3; no worms were found in the lungs at



Graph 4—Mean respiratory rates, group 4.

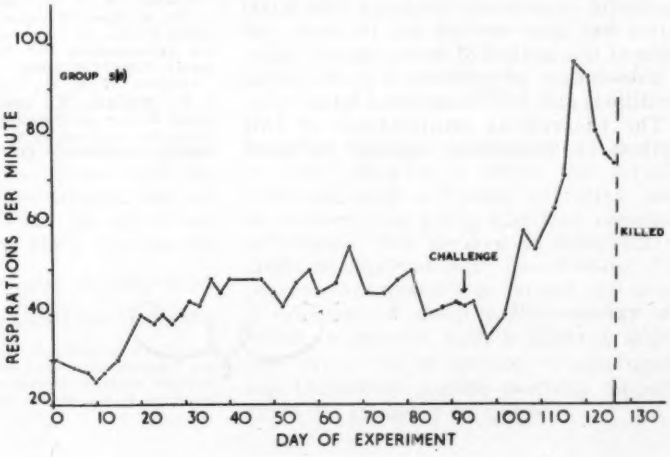


Graph 5—Mean respiratory rates, group 5A.

necropsy and no larvae appeared in the feces of these animals. In group 4, patent infections developed and a mean of 820 worms was found at necropsy after challenge; this result, however, must be interpreted in the light of the pathological findings. In groups 5A and 5B, severe infections developed; it was impossible to assess the total number of worms present in group 5A, as many were immature and were visible only on histological examination.

Pathological Results.—In groups 1, 2, and 3, there were virtually no lesions at necropsy, and histological examination of the lungs failed to show any signs of an infection having been established. In group 4, the lesion score was almost the same as that in groups 5A and 5B, indicating that, macroscopically, the degree of consolidation was the same in the group given one dose of vaccine and in the challenge controls. However, histological examination showed the lesions to be qualitatively different in the two groups. Microscopic examination of sections from calves in group 4 revealed that the majority of the worms in the bronchi were dead or moribund; the lesions produced were those of marked eosinophil leukocyte infiltration into the bronchi and desquamation of the bronchial epithelium.

There was little evidence of alveolar consolidation of the type normally found in lungworm infections. The collapsed lobules, which accounted for the high lesion score, were caused by bronchial plugging with degenerate eosinophil pus. This atypical, ex-



Graph 6—Mean respiratory rates, group 5B.

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TABLE 2—Total Group Weights and Percentage Weight Gains

Group	Total group weights at challenge (lb.)	Total group weights at slaughter (lb.)	Weight gains (%)
1	2,720	3,227	18.6
2	2,632	3,110	18.2
3	2,636	3,092	17.3
4	2,457	2,664	7.6
5	2,607	2,738	5.0

aggerated response to worms dying *in situ* in the lungs has been previously described.[4-6] It is known to resolve in a much shorter time than the lesions produced by a normal infestation by *D. viviparus*.[2] The severe pneumonias recorded in groups 5A and 5B are in keeping with our previous experience of the experimental disease.

DISCUSSION

The results given above show that the administration of two doses of infective larvae of *D. viviparus*, partially inactivated by irradiation, produces a level of immunity which enables calves to withstand a high challenge infection. The clinical effects of the vaccination procedure are mild and would pass unnoticed under practical farming conditions. The difference in weight gain between vaccinated and nonvaccinated animals is significant. No difference was found in response to challenge between those calves which were given 1,000 larvae followed by 1,000 as a vaccinating dose and those given 1,000 followed by 4,000. This may be only a function of the challenge dose; had the latter been higher, differences between the groups might have resulted. A successful experiment involving over 8,000 calves has been carried out to assess the value of this method of immunization under a wide variety of commerical stock-raising conditions and will be reported later.

The theoretical implications of this method of vaccinating against helminth parasites have been discussed elsewhere.[3, 6] It is hoped that this technique might provide a suitable answer to the problem of preventing parasitic bronchitis in calves and ultimately of ridding farms of infection.

SUMMARY

1) A method of artificially immunizing calves against infection with *D. viviparus* is described. The vaccine consisted of third-stage larvae, partially inactivated by exposure to 40,000 roentgens from an x-ray source.

2) The vaccine was given to three groups of calves in two doses; all calves were given 1,000 larvae as the first dose and 4,000, 2,000, and 1,000, respectively, as the second dose.

3) All of the vaccinated calves were completely resistant to infection when challenged with 10,000 normal larvae.

4) The use of this vaccine to prevent the disease in the field is discussed briefly.

References

[1] Jarrett, W. F. H., Jennings, F. W., Martin, B., McIntyre, W. I. M., Mulligan, W., Sharp, N. C. C., and Urquhart, G. M.: A Field Trial of a Parasitic Bronchitis Vaccine. Vet. Rec., *70*, (1958): 451–454.

[2] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: The Natural History of Parasitic Bronchitis with Notes on Prophylaxis and Treatment. Vet. Rec., *69*, (1957): 1329–1337.

[3] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Irradiated Helminth Larvae in Vaccination. Proc. Roy. Soc. Med., *51*, (1958): 743–744.

[4] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Immunological Studies on *Dictyocaulus Viviparus* Infection. The Immunity Resulting from Experimental Infection. Immunology (1959): In press.

[5] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Immunological Studies on *Dictyocaulus Viviparus* Infection. Active Immunisation with Whole Worm Vaccine. Immunology (1959): In press.

[6] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Immunological Studies on *Dictyocaulus Viviparus* Infection. The Immunity Produced by the Administration of Irradiated Larvae. Immunology (1959): In press.

Studies on Immunity to *Haemonchus Contortus* Infection—Vaccination of Sheep Using a Single Dose of X-Irradiated Larvae

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A SUCCESSFUL METHOD of vaccination against the bovine lungworm, *Dictyocaulus viviparus,* has been developed in which the immunizing agent is third-stage infective larvae, partially inactivated by irradiation with either x rays or *gamma* rays from cobalt 60.[2,4,5,8] This technique depends on the fact that lungworm larvae treated with a suitable amount of irradiation migrate to the mesenteric nodes; there, their development appears to be arrested and an antigenic effect is exerted. No significant pathogenic effect takes place because the great majority of the worms do not reach the pulmonary system.

It would appear reasonable to suppose that this method of artificially inducing immunity in parasitic disease might be successful where a systemic migration takes place and where, therefore, the immature larvae have to traverse a potential humoral and cellular immunological barrier; in other diseases in which the parasite undergoes only a limited migration, such as some of the gastrointestinal nematode infections, this method of immunizing might be expected to be less effective. The present experiment was carried out to see if resistance of sheep to *Haemonchus contortus,* a parasite with a minimal migratory cycle, can be induced by administering third-stage larvae partially in activated by exposure to x-rays.

TABLE 1—The Roentgen and Larval Levels Used in Immunizing Doses and the Times of Administering Larvae and Killing Lambs

Group	Roentgen dosage	No. of lambs	Day 0	Day 37	Day 117	Day 163
1 (A)	10,000	2	10,000 larvae	Killed	.....	.....
(B)	10,000	5	10,000 larvae	.....	8,000 normal larvae	Killed
2 (A)	20,000	2	10,000 larvae	Killed	.....	.....
(B)	20,000	5	10,000 larvae	.....	8,000 normal larvae	Killed
3 (A)	40,000	2	10,000 larvae	Killed	.....	.....
(B)	40,000	5	10,000 larvae	.....	8,000 normal larvae	Killed
4 (A)	60,000	2	10,000 larvae	Killed	.....	.....
(B)	60,000	5	10,000 larvae	.....	8,000 normal larvae	Killed
5 (A)	100,000	2	10,000 larvae	Killed	.....	.....
(B)	100,000	5	10,000 larvae	.....	8,000 normal larvae	Killed
6	.....	5	10,000 larvae	Killed	.....	.....
7	.....	5	10,000 larvae	.....	8,000 normal larvae	Killed
8	.....	5	.....	.....	8,000 normal larvae	Killed

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TABLE 2—Parasitological Findings in Lambs Killed on Day 37

Group	Eggs per gram of feces: Day 18	Eggs per gram of feces: Day 33	Worm count at necropsy: Immature	Worm count at necropsy: Mature	Group means
1 (A)	+*	3,100	.....	1,900	
	+	1,100	.....	3,700	2,800
2 (A)	+	1,400	.....	1,850	
	+	1,500	.....	1,500	1,675
3 (A)	+	0	300	0	
	0	0	200	0	250
4 (A)	0	50	400	0	
	0	50	100	0	250
5 (A)	+	50	100	0	
	0	0	0	0	50
	+	1,100	0	1,100	
	+	3,300	0	2,700	
6	+	2,000	0	1,600	1,900
	+	1,850	0	2,400	
	+	2,050	0	1,700	

* Positive on nonquantitative zinc sulfate flotation only.

MATERIALS AND METHODS

Fifty newborn lambs were bought and initially were raised in groups of 3 or 4 on an artificial milk diet* in metal isolation units. They were later transferred to concrete pens which were cleaned daily. Their feces were examined several times in the pre-experimental period to ensure that they were parasite free. The lambs were 7 months old when the experiment was started.

A pure strain of *H. contortus* was obtained† and passaged through 3 worm-free lambs to get a sufficient number of infective larvae to carry out the experiment. Larvae were cultured by putting 250 ml. of feces in a 500-ml. jar, storing at 22 C. for 14 days, filling the jar with water, and inverting it over a Petri dish. The larvae were obtained from the lower 100 ml. of water. Fecal egg counts were estimated by the McMaster method and total worm burdens by washing out the abomasal contents, making up to 2,000 ml. in tap water, stirring thoroughly, and counting the number of worms in 150 ml.

The technique of irradiation was as used previously for *D. viviparus*.[8]

EXPERIMENTAL PROCEDURE

The lambs were allotted to eight groups numbered 1 to 8. There were 7 lambs each in groups 1 to 5, and 5 lambs each in groups 6 to 8. On day zero, animals in groups 1 through 5 were given 10,000 infective larvae, irradiated respectively with 10,000, 20,000, 40,000, 60,000, and 100,000 roentgens. Two lambs from each of the groups were killed at day 37 to determine the degree of inactivation of larvae achieved. The remainder were challenged on day 117 with 8,000 normal infective larvae and were killed on day 163.

* Ostermilk, produced by Messrs. Glaxo Laboratories, Ltd., Greenford, Middlesex, England.

† Supplied by Drs. Dunn and Silverman of the Moredun Research Institute, Gilmerton, Edinburgh.

TABLE 3—Parasitological Findings in Lambs of Group 1 (B)

Lamb No.	Eggs per gram of feces on day: 18	34	40	48	54	61	68	76	83	90	96	103	110	117	144*	146	158	No. of worms at necropsy
1	++	300	1,350	2,050	2,450	1,850	1,800	800	500	0	0	0	0	0	20	0	0	0
2	0	4,400	700	1,000	500	500	0	n.s.**	100	0	0	0	0	0	0	0	0	0
3	+	2,500	1,200	4,300	5,800	3,050	7,000	2,000	2,100	3,100	3,350	2,000	1,500	100	0	40	300	20
4	+	2,000	3,000	1,650	1,000	1,200	0	200	50	0	50	0	0	0	0	0	0	0
5	0	2,450	2,100	0	50	0	0	0	0	40	0	0	0	0	0	0	0	7
Mean		2,200	1,670	1,800	1,960	1,280	1,720	750	550	630	680	400	300	20	4	8	60	5

TABLE 4—Parasitological Findings in Lambs of Group 2 (B)

Lamb No.	Eggs per gram of feces on day: 18	34	40	48	54	61	68	76	83	90	96	103	110	117	144*	146	158	No. of worms at necropsy
1	++	1,850	2,900	1,400	2,650	2,700	2,550	1,100	800	0	50	0	0	0	0	0	0	0
2	+	1,100	2,850	2,100	1,700	1,100	750	800	800	2,000	0	0	0	0	0	0	0	0
3	0	2,050	1,150	450	500	350	50	0	50	50	0	0	0	0	0	0	0	0
4	0	100	1,300	2,500	800	2,600	3,000	1,900	600	0	0	0	0	0	0	0	0	0
5	+	2,150	2,400	2,100	100	0	0	0	0	0	0	0	0	0	0	0	50	0
Mean		1,500	2,120	1,710	1,150	1,350	1,270	760	580	530	10	0	0	0	0	0	10	0

* Point of challenge; † positive on nonquantitative zinc sulfate flotation only; ** no sample obtained.

TABLE 5—Parasitological Findings in Lambs of Group 3 (B)

Lamb No.	Eggs per gram of feces on day:																	No. of worms at necropsy
	18	34	40	48	54	61	68	76	83	90	96	103	110	117	144*	146	158	
1	+†	150	50	0	50	0	20	50	20	0	0	0	0	0	0	0	100	972
2	0	100	50	0	0	0	20	n.s.**	0	0	0	0	0	0	0	0	0	0
3	0	0	150	100	0	40	0	0	0	n.s.	0	0	20	0	0	0	50	0
4	0	150	0	150	50	100	0	0	0	n.s.	0	0	0	0	0	0	0	0
5	0	0	150	n.s.	50	0	0	0	0	n.s.	0	0	0	0	0	20	0	40
Mean		60	80	50	30	30	8	10	5	0	0	0	4	0	0	4	30	200

* Point of challenge; † positive on nonquantitative zinc sulfate flotation only; ** no sample obtained.

Each lamb in group 6 was given 10,000 normal larvae on day zero. All lambs were killed on day 37 as a control of the infectivity of the larvae used to make the vaccine.

Each lamb of group 7 was given 10,000 normal larvae on day zero, 8,000 normal larvae on day 117, and was killed on day 163.

Group 8 was the challenge control group; each lamb received 8,000 normal larvae on day 117 and was killed on day 163 (table 1).

RESULTS

Counts of fecal egg output were made at approximately weekly intervals beginning on day 18; these results, together with the postmortem abomasal worm burdens are shown (tables 2–9). The final results of the experiment are summarized (table 10).

The lambs did not show clinical signs of disease at any time during the experiment.

TABLE 6—Parasitological Findings in Lambs of Group 4 (B)

Lamb No.	Eggs per gram of feces on day:																	No. of worms at necropsy
	18	34	40	48	54	61	68	76	83	90	96	103	110	117	144*	146	158	
1	+†	50	0	0	0	0	0	0	0	n.s.**	50	20	0	0	0	0	0	0
2	+	0	0	0	0	0	0	0	0	n.s.	20	0	0	0	0	0	50	0
3	0	0	0	0	0	0	0	0	0	n.s.	20	0	0	0	0	0	0	0
4	0	0	0	0	0	100	20	0	0	n.s.	0	0	0	0	0	0	0	0
5	0	0	100	0	0	0	0	0	0	n.s.	0	0	0	0	0	0	0	0
Mean		10	20	0	0	20	4	0	0	.....	18	4	0	0	0	0	10	0

* Point of challenge; † positive on nonquantitative zinc sulfate flotation only; ** no sample obtained.

DISCUSSION

In assessing the value of this method of making a vaccine, the first point to be decided is whether the particular parasite can be partially inactivated and, if so, what is the limiting range of radiation dosage to be applied to it. Two animals from each of the vaccine groups were killed when fecal counts of the control lambs (group 6) showed that the normal infections had reached full patency (table 2). Lambs in groups 1A and 2A showed no significant reduction in "take" over the control animals, indicating that the larvae were insufficiently irradiated; lambs in groups 3A, 4A, and 5A did have a markedly lower worm burden and showed that these radiation dosages possibly encompassed the optimal level. In the light of the findings of the challenge infection of animals in group 5B (given larvae treated with 100,000 roentgens), in which there was evidence of only a moderate degree of immunity, it would appear that the optimal level of irradiation lies between 40,000 roentgens and 60,000 roentgens, since lambs in groups 3 and 4 showed marked inactivation of larvae and also good immunity. It is of interest that the 60,000-roentgen dose produces too much inactivation in the case of *D. viviparus* larvae and that these do not penetrate far enough to produce immunity, whereas we have found that

TABLE 7—Parasitological Findings in Lambs of Group 5 (B)

Lamb No.	Eggs per gram of feces on day:																	No. of worms at necropsy
	18	34	40	48	54	61	68	76	83	90	96	103	110	117	144*	146	158	
1	0	50	0	0	0	0	0	0	0	n.s.†	0	0	0	0	0	100	10,000	1,540
2	0	0	0	0	0	0	0	0	0	n.s.	0	0	0	0	0	50	2,800	397
3	+**	0	0	0	0	0	0	0	0	n.s.	0	0	0	0	0	0	50	270
4	+	0	0	0	0	0	20	0	0	n.s.	20	0	0	0	0	0	0	0
5	0	0	0	0	50	0	0	0	0	n.s.	0	0	0	0	0	0	100	0
Mean		10	0	0	10	0	4	0	0	.....	4	0	0	0	0	30	2,590	442

* Point of challenge; † no sample obtained; ** positive on nonquantitative zinc sulfate flotation only.

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TABLE 8—Parasitological Findings in Lambs of Group 7

Lamb No.	18	34	40	48	54	Eggs per gram of feces on day: 61	68	76	83	90	96	103	110	117	144*	146	158	No. of worms at necropsy
1	+†	1,000	300	150	600	150	900	0	0	0	0	0	0	0	0	0	0	0
2	+	1,000	2,100	450	800	1,550	300	0	0	0	40	0	0	0	0	20	4,000	1,450
3	+	3,850	2,500	700	2,050	850	150	100	350	350	0	0	0	0	0	0	600	1,500
4	0	900	1,700	1,000	4,200	3,750	4,200	2,000	900	0	0	0	0	0	0	0	n.s.**	150
5	+	2,450	4,200	250	6,450	7,600	4,200	1,800	300	60	150	20	0	0	0	0	0	0
Mean		2,000	2,160	510	2,820	2,760	1,950	780	310	82	40	4	0	0	0	4	1,150	620

* Point of challenge; † positive on nonquantitative zinc sulfate flotation only; ** no sample obtained.

TABLE 9—Parasitological Findings in Lambs of Group 8

Lamb No.	Eggs per gram of feces on day: 144	146	158	No. of worms at necropsy
1	0	0	2,350	1,550
2	0	0	8,850	2,390
3	0	0	4,400	430
4	0	0	1,850	4,240
5	0	0	2,250	1,600
Mean	0	0	3,940	2,042

doses as low as 5,000 roentgens are sufficient to inactivate *Fasciola gigantica.*

The conclusions which can be drawn from this experiment are limited but encouraging, and may be summarized as follows: (1) within the experimental system used (relatively small numbers of lambs, larvae given as one single dose, subclinical levels of infection and a relatively short time between vaccination and challenge), lambs develop a degree of resistance to reinfection by *H. contortus;* (2) it is possible to partially inactivate third stage infective *H. contortus* larvae by x-irradiation; and (3) such larvae, if subjected to a dose of x-rays between 40,000 and 60,000 roentgens, will produce a marked resistance to reinfection.

If these results are confirmed by later work using a wider range of challenge doses, they have an important bearing on certain fundamental factors in helminth immunity. They indicate that the minimal penetration and short period of larval development is sufficient to stimulate an appreciable immune response and that the presence of adults may not be necessary to produce resistance. From earlier work [1,2] with *Trichinella spiralis,* it has been suggested [10] that adult infections are necessary to induce refractoriness to reinfection with gastrointestinal nematodes. This does not appear to be the case in *D. viviparus* infection [2,4,5,7,8] and the present results indicate that *H. contortus,* a purely alimentary worm, may also fail to conform to this theory.

TABLE 10—Summary of Results of Challenge Infection

Group	Roentgen dose	Mean No. of worms on challenge
1 (B)	10,000	5
2(B)	20,000	0
3 (B)	40,000	200
4 (B)	60,000	0
5 (B)	100,000	442
7	Double infection	620
8	Control	2,042

If larvae can be partially inactivated by this method, and yet induce immunity, this would appear to be a fruitful field of research in many helminth diseases. Little is known of the quantitative aspects of immune response to helminth parasites, but in bovine parasitic bronchitis it is known [6] that a typical anamnestic response takes place after reinfection. We have also shown,[3] in parasitic bronchitis, that if calves are given two doses of irradiated larvae with a 30-day interval between, they can completely withstand a challenge infection which is lethal to untreated susceptible animals. An experiment is in progress to asses the value of this double vaccination procedure in *H. contortus* infections.

SUMMARY

Batches of infective larvae of *Haemonchus contortus* were treated with, respectively, 10,000, 20,000, 40,000, 60,000, and 100,000 roentgens of x-rays. The larvae were then given orally to sheep to assess the degree of inactivation achieved by irradiation and the immunity produced by such treated larvae. It was found that larvae subjected to 40,000 and 60,000 roentgens produced a good immunity to reinfection. The applications of these findings to vaccination against the parasite are discussed.

References

[1] Gould, S. E., Gomberg, H. J., Bethell, F. H., Gillella, J. B., and Hertz, C. S.: Studies on *Trichinella Spiralis*. Am. J. Path., *31*, (1955): 933–963.

[2] Jarrett, W. F. H., Jennings, F. W., Martin, B., McIntyre, W. I. M., Mulligan, W., Sharp, N. C. C., and Urquhart, G. M.: A Field Trial of a Parasitic Bronchitis Vaccine. Vet. Rec., *70*, (1958): 451–454.

[3] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., Sharp, N. C. C., and Urquhart, G. M.: Vaccination Against *Dictyocaulus Viviparus* Infection in Calves—Double Vaccination with Irradiated Larvae. Am. J. Vet. Res., *20*, (May, 1959): 522–526.

[4] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: The Natural History of Parasitic Bronchitis with Notes on Prophylaxis and Treatment. Vet. Rec., *69*, (1957): 1329–1337.

[5] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Irradiated Helminth Larvae in Vaccination. Proc. Roy. Soc. Med., *51*, (1958): 743–744.

[6] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Immunological Studies on *Dictyocaulus Viviparus* Infection. The Immunity Resulting from Experimental Infection. Immunology, (1959): In press.

[7] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., Urquhart, G. M.: Immunological Studies on *Dictyocaulus Viviparus* Infection. Active Immunisation with Whole Worm Vaccine. Immunology, (1959): In press.

[8] Jarrett, W. F. H., Jennings, F. W., McIntyre, W. I. M., Mulligan, W., and Urquhart, G. M.: Immunological Studies on *Dictyocaulus Viviparus* Infection. The Immunity Produced by the Administration of Irradiated Larvae. Immunology, (1959): In press.

[9] Levin, A. J., and Evans, T. C.: The Use of Roentgen Radiation in Locating an Origin of Host Resistance to *Trichinella Spiralis* Infections. J. Parasitol., *28*, (1942): 477–483.

[10] Soulsby, E. J. L.: Some Immunological Phenomena in Parasitic Infections. Vet. Rec., *69*, (1957): 1129–1139.

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Biochemical and Serological Separation of Some Members of the Genus Vibrio

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VIBRIOS ARE widely distributed in nature; they may be found in water, soil, and animal and human hosts. Difficulty is often encountered when these organisms must be characterized. In this study, emphasis was placed on vibrios which are known to be pathogenic primarily for domestic animals. *Vibrio comma* and certain saprophytic Vibrio species were included for comparison. Data obtained from a study of the biochemical and serological characteristics of diffierent vibrios from various sources are discussed.

REVIEW OF LITERATURE

The first isolation of a Vibrio from animals was made by Gamalèia[7] in 1888 in Odessa, Russia. The organism, closely related to the cholera Vibrio both morphologically and physiologically, was isolated from chickens dying of acute intestinal infections.

Winter dysentery was first described in the United States by Steffen[21] in 1915. Smith and Orcutt[19] studied the serological relation of *Vibrio fetus* and vibrios isolated from calves suffering from acute enteritis. In 1931, Jones and Little[11] described the etiology, pathology, and clinical features of the disease, and Jones[10] named the organism *Vibrio jejuni*. The disease has been described in England, Sweden, Germany, and other countries.

Vibrionic dysentery in swine was first recognized in this country by Hofferd[9] in 1918. In 1924, Whiting[23] reported reproducing the disease experimentally by feeding healthy animals infected material from diseased swine. Extensive studies of the disease were reported by Doyle in a series of papers[3-5] from 1943 to 1948. He isolated a Vibrio from affected animals and successfully reproduced the disease in healthy animals. He named the organism *Vibrio coli*. At least two distinct morphological types of vibrios were recognized in affected colons. Doyle suggested that one form may occur as a normal inhabitant of swine intestine. Harms and Hoiter[8] described methods of cultivating the organism. The biochemical and serological properties of different vibrios occurring in swine are not well known, and their relationship to other animal pathogens is not clear.

Vibrionic abortion was first observed in Great Britain in 1909 by McFadyean and Stockman.[18] A Vibrio was isolated from the aborting ewes. A decade later in this country, Smith[18] was the first to report vibrionic abortion in cattle which were free of brucellosis. The organism was named *V. fetus* by Smith and Taylor,[20] since at that time it could be isolated only from aborted fetuses.

In cattle, the disease is mainly one of temporary infertility with abortion being secondary.[22] Vibriosis in sheep manifests itself as an abortion disease.[14]

Evidence given by many investigators shows that vibrios, other than *V. fetus*, inhabit the bovine and ovine genital tract. Little work has been done to systematically characterize this group of vibrios. Frank and Bryner[6] found these organisms to be catalase-negative, H_2S-positive, and morphologically indistinguishable from *V. fetus*. Since these organisms are indistinguishable morphologically from *V. fetus*, biochemical and serological tests should be used for their differentiation. Price *et al.*[17] found that vibrios from nonfetal sources which were H_2S-positive and serologically unrelated to fetal strains were not *V. fetus*.

Kuzdas and Morse[13] studied biochemical characteristics of different Vibrio cultures and found that *V. fetus*, irrespective of source of isolation, was defined as catalase-positive and H_2S-negative. Human strains of *V. fetus* were closely related biochemically to animal strains.

King[12] studied the public health significance of *V. fetus* strains. Vibrios isolated in cases of gastroenteritis in man were found to be serologically related to *V. fetus* and were termed the "related Vibrio" group. Possible serological relationships between *V. jejuni* and *V. fetus*, or to members of the related Vibrio group, were suggested.

MATERIALS AND METHODS

Cultures.—The 68 Vibrio cultures used in this study were of bovine, ovine, human, porcine, and avian origin. These cultures included *V. fetus* strains, catalase-negative vibrios, members of the related Vibrio group, and swine vibrios. Other

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vibrios—*V. comma*, *V. jejuni* (ATCC 11734), *V. metschnikovii* (ATCC 7708), *V. cuneatus* (ATCC 6972), *V. percolans* (ATCC 8461), *V. proteus* (ATCC 6361), and *V. tyrogenus* (ATCC 7085)—were obtained from the American Type Culture Collection.

Cultures were isolated according to the methods of Bryner and Frank.[2] Prior to biochemical and serological studies, all cultures were examined for smooth, rough, and mucoid colonies according to the methods used by Bond.[1] Smooth colonies were used for biochemical and serological tests. The inoculum used in all biochemical tests was 0.2 ml. of a well-mixed 48- to 72-hour thioglycollate culture.

Stock cultures were maintained in thioglycollate medium (BBL 01-135C) and transferred at biweekly intervals. Cultures of *V. comma* were maintained on heart-infusion agar slants adjusted to pH 8.0.

Biochemical Tests.—Catalase production was determined by adding 2 ml. of 3 per cent H_2O_2 solution to 7 ml. of a 48- to 72-hour thioglycollate culture. The mixture was shaken and placed in a water bath at 37 C.

Hydrogen sulfide was detected by the lead acetate test-strip technique. Brain-heart infusion (BBL 01-239), containing 0.7 Gm. of agar and 0.3 Gm. of cysteine HCl per liter, was used as the test medium.

Ability to reduce nitrates was demonstrated by inoculating cultures into thioglycollate medium containing 1 Gm. of KNO_3 per liter; nitrite was detected by recommended methods.[15]

Indole production was determined by the Gnezda technique. Indole-nitrite medium (BBL 01-155) was used.

Salt-tolerance tests were performed by inoculating cultures into thioglycollate medium containing 1, 3, and 5 per cent salt. Cultures were incubated at 37 C. for five days.

Cultures were inoculated into nutrient broth (BBL 01-125) and incubated at 37 C. for five days. Growth was compared with that occurring in thioglycollate medium.

Cultures were tested in litmus milk and trypticase-enriched litmus milk. The latter was prepared by adding 10 Gm. of trypticase per liter of litmus milk. Litmus milk cultures were incubated at 37 C. for 14 days.

Sensitivity to methylene blue was determined by inoculating tubes of thioglycollate medium containing 0.01 and 0.05 per cent methylene blue. These tubes were incubated at 37 C. for three to five days. Reduction of the dye was used as the criterion for growth. In doubtful reactions, microscopic examinations were made to determine growth.

The pH values of five-day thioglycollate cultures were determined on the Photovolt (model 115) pH meter.

The cholera-red reaction was performed by adding slowly 1 ml. of concentrated H_2SO_4 to 7 ml. of a 48-hour culture grown in indole-nitrite medium.

The ability to ferment 22 carbohydrates was determined by inoculating cystine-trypticase agar (BBL 01-174) deeps containing 1 per cent carbohydrate. Inoculated tubes were incubated at 37 C. for five days. Suitable controls were checked simultaneously with the performance of each test.

Serology.—Antigens were grown on thioglycollate agar (2.5%) in Kolle flasks. This medium was suitable for antigen production, since it produced good growth of most cultures except *V. comma* and the catalase-negative vibrios. Antigens prepared in this manner exhibited cells of uniform morphology and active motility which were not autoagglutinable in physiological saline solution.

Flasks were incubated under 10 per cent CO_2 for three to five days. *Vibrio comma* antigen was grown on heart-infusion agar slants adjusted to pH 8.0. Catalase-negative vibrios were grown in thioglycollate medium from which all traces of agar were removed. All antigens were washed in sterile physiological saline solution (0.85%) three times at 3,500 r.p.m. for 15 minutes. Concentrated antigens were stored at 5 C.

Antiserums were prepared by injecting 5- to 7-lb. rabbits intravenously with living cultures (except *V. comma*). Antigens were adjusted to a density corresponding to No. 3 on the McFarland nephelometer scale. This antigen was injected in 0.5 ml., 1.0 ml., and 1.5 ml. amounts for three successive days. After a 4-day lapse, antigens were adjusted to McFarland No. 4 and administered in 0.5, 1.0, 1.5, and 2.0 ml. amounts for four successive days. Six days after the last injection, trial blood samples were taken to determine antibody titer. Animals with sufficient titers were exsanguinated.

Prior to immunization, 5 ml. of blood was withdrawn from each rabbit for the detection of normal agglutinins.

Immune serums were dispensed in 2.0-ml. quantities and preserved at −60 C. Repeated freezing and thawing of serum did not significantly alter antibody titer. The reciprocal whole-cell cross-agglutination test was used to determine serological relationships between 68 antigens and 59 antiserums. Dilutions were made according to the method used by Price *et al.*[17] Final serum dilutions were 1:50 to 1:6,400.

All tube agglutination antigens were standardized by diluting with formolized saline solution (0.3%) to a final concentration of cells equal to about 4.5 by 10^9 per milliliter. A reading of 75 per cent transmittance using the 440 mμ filter of the Brociner-Mass Clinical Analyzer gave a suspension of the desired density. In each tube, 2 ml. of diluted antigen was used. The tubes were incubated at 37 C. for 48 hours, then observed for the nature and size of the clump and the amount of clearing occurring in the antigen suspension. The antibody titer was expressed as the reciprocal of the highest serum dilution showing a 3+ agglutination.

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Results and Discussion

Biochemical and serological reactions of 68 different Vibrio cultures from various sources were studied. Cultures were divided into groups on the basis of the catalase reaction or the animal host from which they were isolated, or both. Group 1 contained bovine catalase-positive cultures; group 2 contained bovine, ovine, and human catalase-negative cultures; group 3 contained ovine *V. fetus* cultures; group 4 contained human *V. fetus* cultures; group 5 contained cultures of the related Vibrio group isolated from human beings with cases of gastroenteritis; group 6 represented a *V. comma* strain used; group 7 contained vibrios of swine origin; and group 8 contained *V. metschnikovii* and four saprophytic species.

The only cultures which fermented carbohydrates were *V. comma, V. metschnikovii, V. cuneatus,* and *V. proteus.* Fermentation reactions were not valuable for differentiation of other Vibrio cultures. Results obtained with methylene blue sensitivity tests were not definite enough to permit differentiation. However, bovine strains of *V. fetus* (group 1) appeared to be more sensitive to methylene blue than ovine and human strains were.

Growth in nutrient broth did not reveal marked differences for characterizing these cultures. However, *V. fetus* cultures of bovine origin (group 1) and members of the related Vibrio group (group 5) did not grow as well as other cultures.

The cholera-red reaction failed to differentiate these vibrios, since most of the cultures were incapable of forming indole and reducing nitrate simultaneously; *V. comma* and *V. metschnikovii* gave strong cholera-red reactions. In certain indole-negative and nitrite-positive cultures, cholera-red reactions were observed. Further work is needed to explain these results.

Several routine biochemical tests for the differentiation of the vibrios are the catalase reaction, H_2S production, nitrate reduction, salt-tolerance, litmus milk reactions, and pH of thioglycollate medium. Emphasis was placed on these reactions as produced by the different groups of vibrios in light of the serological relationships which existed between the groups.

The following results were obtained from agglutination reactions when 68 vibrio antigens were crossed with 59 prepared antiserums. In general, *V. fetus* strains (groups 1, 3, and 4) appeared to be closely related serologically, with the exception of three cultures. Bovine *V. fetus* cultures (group 1) were catalase-positive and usually H_2S-negative. The cultures reduced nitrates strongly and failed to grow in the presence of 3 per cent salt or produce a change in plain litmus milk. An alkaline reaction (with some reduction of litmus) was observed in enriched litmus milk and in five-day thioglycollate cultures. In contrast, *V. fetus* cultures of ovine (group 3) and human (group 4) origin were biochemically similar to bovine strains, but differed in that they produced small quantities of H_2S and some degree of peptonization in litmus milk. Peptonization was never observed with bovine strains. *Vibrio jejuni* (group 1) was serologically distinct from all *V. fetus* strains (groups 1, 3, and 4). The organism also differed by its failure to reduce nitrate.

The most distinguishing biochemical characteristics of vibrios in group 2 were the catalase and H_2S reactions, salt-tolerance and pH value of five-day cultures. These cultures were catalase-negative, H_2S-positive, tolerant to 3 per cent (sometimes 5%) salt, and unable to produce a pH of more than 7.5 in five-day cultures. On the basis of cross-agglutination reactions these organisms, with one exception, appeared to be serologically unrelated to all other cultures. The exception was a Vibrio of human origin.

The related Vibrio group (group 5) included vibrios isolated from human beings with cases of gastroenteritis. These organisms appeared to be serologically related to many *V. fetus* cultures (groups 1, 3, and 4). However, certain biochemical differences may be pointed out which might serve to distinguish these vibrios from *V. fetus.* Related Vibrio cultures were catalase-positive, H_2S-positive, and produced an alkaline curd with reduction of litmus in enriched litmus milk. King[12] observed that these vibrios might be differentiated further by their ability to grow luxuriantly at 42 C. Serological relationships could not be demonstrated between *V. jejuni* and members of the related Vibrio group. The possible relationship of this organism to related Vibrio cultures has been mentioned by King.

Six cultures of the swine Vibrio group (group 7) appeared to be serologically and

biochemically heterogeneous. On the basis of serological reactions, the group was divided into three subgroups. The first subgroup contained one culture which was serologically distinct. The second subgroup contained two cultures which were serologically related to each other. These two subgroups appeared closely related biochemically to members of the related Vibrio group (group 5), but failed to curdle litmus milk. It is possible that these organisms may be *V. coli*. Unfortunately, this was not confirmed, since known *V. coli* cultures were unavailable for comparison. The third subgroup contained three cultures which were serologically related to each other and to *V. fetus*. Biochemically, these cultures differed from *V. fetus* in that they were H_2S-variable and salt-tolerant. Detection of *V. fetus*-like organisms in swine indicates that these animals may serve as reservoirs of *V. fetus* infection in other livestock. Strains isolated from cattle, sheep and, possibly, human infections may merely represent swine vibrios (daughter strains) which have adapted to other animal hosts. Identification of swine vibrios may be made by using biochemical and serological tests simultaneously.

The four saprophytic Vibrio species (group 8) were serologically distinct species. Two species, *V. cuneatus* and *V. proteus*, were capable of fermenting carbohydrates, while *V. percolans* and *V. tyrogenus* failed in this respect. *Vibrio proteus* was the only saprophytic species unable to split urea. All saprophytic species liquefied gelatin except *V. percolans*.

Vibrio comma and *V. metschnikovii* were serologically distinct species. These two vibrios were relatively active biochemically. Liquefaction of gelatin, indole production, salt tolerance, and litmus milk reactions identified and differentiated these species from vibrios found in animal hosts. *V. metschnikovii* was differentiated from *V. comma* by its ability to curdle litmus milk.

SUMMARY

Biochemical and serological reactions of 68 vibrio cultures from various sources were studied.

Vibrio fetus cultures of bovine, ovine, and human origin were serologically related (groups 1, 3, and 4).

Bovine strains of *V. fetus* (group 1) were catalase-positive, usually H_2S-negative, salt-sensitive, and negative in plain litmus milk. They produced an alkaline reaction with reduction in enriched litmus milk and raised the pH (pH 8.0) of thioglycollate medium after five days of incubation. Several cultures in group 1 did not conform biochemically or serologically, or both. Ovine (group 3) and human (group 4) strains of *V. fetus* were less fastidious than bovine strains, were weakly positive for H_2S, and peptonized enriched litmus milk.

Vibrio jejuni (group 1) was serologically distinct and failed to reduce nitrate.

Vibrio fetus strains (groups 1, 3, and 4), irrespective of source, appeared to be (with one exception) the same organism serologically, with minor biochemical differences.

Vibrios from nonfetal sources (group 2), which were serologically unrelated to *V. fetus*, were catalase-negative, H_2S-positive, salt-tolerant, and unable to raise the pH of thioglycollate medium after five days of incubation. Members of the catalase-negative group were serologically related to each other, with one exception.

Related Vibrio cultures (group 5) were serologically related to *V. fetus* cultures. Biochemically, they differed from *V. fetus* by producing large quantities of H_2S and curdling litmus milk.

The presence of three serological subgroups in group 7 (swine vibrios) was indicated. The group was not homogeneous on the basis of biochemical and serological reactions. The isolation of *V. fetus*-like organisms from swine indicated the possibility of these animals being carriers for *V. fetus* infection in cattle, sheep, goats and, possibly, in man.

Vibrio comma, *Vibrio metschnikovii*, and four saprophytic Vibrio species were easily differentiated from vibrios found in livestock.

References

[1] Bond, J. M.: Characteristics of Colonial Forms of *Vibrio Fetus*. Am. J. Vet. Res., *18*, (April, 1957): 449–455.

[2] Bryner, J. H., and Frank, A. H.: Laboratory Techniques for Isolation and Propagation of Vibrio from Cattle. Am. J. Vet. Res., *16*, (Oct., 1955): 634–635.

[3] Doyle, L. P.: Swine Dysentery. J.A.V.M.A., *102*, (June, 1943): 449–451.

[4] Doyle, L. P.: A Vibrio Associated with Swine Dysentery. Am. J. Vet. Res., *5*, (Jan., 1944): 3–5.

[5] Doyle, L. P.: Etiology of Swine Dysentery. Am. J. Vet. Res., *9*, (Jan., 1948): 50–51.

[6] Frank, A. H., and Bryner, J. H.: Observations on Vibriosis of Cattle in Relation to Impaired Fertility. 57th Ann. Proc. U.S. Livestock San. A. (1953): 165–175.

[7] Gamaléia, M. N.: *Vibrio Metschnikovii* (n. sp.). Ann. Inst. Pasteur, *2*, (1888): 482.

XUM

[8] Harms, F., and Hoiter, R.: The Cultivation of *Vibrio Fetus* and *Vibrio Suis*. Deutsche tierärztl. Wchnschr., *60*, (1953): 320–322.

[9] Hofferd, R. M.: Swine Dystentery in Iowa From a Field Standpoint. J.A.V.M.A., *88*, (March, 1936): 299–310.

[10] Jones, F. S., and Little, R. B.: Vibrionic Enteritis in Calves. J. Exptl. Med., *53*, (1931): 845–851.

[11] Jones, F. S., Orcutt, M., and Little, R. B.: Vibrios *(Vibrio Jejuni*, n. sp.) Associated with Intestinal Disorders of Cows and Calves. J. Exptl. Med., *53*, (1931): 853.

[12] King, E. O.: Human Infections with *Vibrio Fetus* and a Closely Related Vibrio. J. Infect. Dis., *101*, (1957): 119–128.

[13] Kuzdas, C. D., and Morse, E. V.: Physiological Characteristics Differentiating *Vibrio Fetus* and Other Vibrios. Am. J. Vet. Res., *17*, (April, 1956): 331–336.

[14] Lindenstruth, R. W., Ashcraft, J. B., and Ward, B. Q.: Studies on Vibrionic Abortion of Sheep. J.A.V.M.A., *114*, (April, 1949): 204–208.

[15] Manual of Microbiological Methods. S.A.B., Committee on Bact. Technique. McGraw-Hill Book Co., Inc., New York, N.Y., 1957.

[16] McFadyean, J., and Stockman, S.: Report of the Departmental Committee on Epizootic Abortion. Great Britain Board of Agriculture and Fisheries, London (1913): 22.

[17] Price, K. E., Poelma, L. J., and Faber, J. E.: Serological and Physiological Relationships Between Strains of *Vibrio Fetus*. Am. J. Vet. Res., *16*, (Jan., 1955): 164–169.

[18] Smith, T.: Spirilla Associated with Disease of the Fetal Membranes in Cattle (Infectious Abortion). J. Exptl. Med., *28*, (1918): 701–719.

[19] Smith, T., and Orcutt, M. L.: Vibrios from Calves and Their Serological Relation to *Vibrio Fetus*. J. Exptl. Med., *45*, (1927): 391–397.

[20] Smith, T., and Taylor, M. S.: Some Morphological and Biological Characters of the Spirilla (*Vibrio Fetus* n. sp.) Associated with Disease of the Fetal Membranes in Cattle. J. Exptl. Med., *30*, (1919): 299–312.

[21] Steffen, M. R.: Special Cattle Therapy. Am. J. Vet. Med., (1915): 56.

[22] Terpstra, J., and Eisma, W. A.: *Vibrio Fœtus* Infection in Cattle and Enzootic Infertility. Tijdschr. Diergeneesk., *76*, (1951): 433–447.

[23] Whiting, R. A., Doyle, L. P., and Spray, R. S.: Swine Dysentery. Purdue Univ. Agric. Exper. Sta. Bull., Oct., 1921.

Electron Microscopic Studies of the Virus of Avian Infectious Laryngotracheitis

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THE VIRUS of avian infectious laryngotracheitis (LT) has been the subject of periodic study since its first identification by Beach.[1,2] The size of viral particles was originally estimated as 45 to 82 mμ in diameter by means of filtration through a graded series of collodion membranes.[15] Recently, Reagan *et al.*[23] employed the electron microscope for their investigations and found that the viral elements in shadowcast preparations ranged from 100 to 110 mμ.

A comprehensive paper describing pathological changes encountered in avian infectious laryngotracheitis was presented by Seifried.[26] The author based his observations on 30 field and experimentally infected cases and found that characteristic lesions occurred in the respiratory system. Most commonly involved were the larynx and trachea, both of which showed a degree of inflammatory reaction, particularly in the mucosa. Microscopically, the affected mucous membrane was edematous and infiltrated with inflammatory cells. Many of the epithelial cells had undergone proliferation, and later in the disease showed degenerative and necrotic changes. Acidophilic, intranuclear inclusion bodies, often separated from the marginated chromatin by a clear zone, were seen in a number of epithelial cells. According to Seifried, most of the inclusions reacted positively to the Feulgen test and, when treated with silver salts, displayed an internal granular structure.

The first successful attempt at propagation of the LT virus on the chorioallantoic membrane of the chicken embryo was reported by Burnet.[7] The lesions produced took the form of opaque plaques and were characterized by hyperplasia, chiefly of the ectodermal cells, and by acute inflammation of the mesoderm. Acidophilic, intranuclear inclusion bodies occurred in a number of the affected cells. The author admitted, however, that "the predominance of ectodermal changes" could be "related mainly to the method of inoculation." Similar findings were recorded by Brandly.[6]

The purpose of this report is to describe the structure of the laryngotracheitis virus and to elaborate on some aspects of the cellular changes encountered in the chorioallantoic membrane of infected chicken embryos.

MATERIALS AND METHODS

The virus used for this study was isolated from a bird with a field case of laryngotracheitis (strain N 71851) and has been maintained in a lyophilized state, with periodic tests for viability, for six years.

Nine-day embryonating chicken eggs were inoculated with 0.2 ml. of a suspension of the virus on the chorioallantoic membrane, according to the technique described by Gorham.[10] After five days of incubation, the shell over the air sac and the inner shell membrane were removed; the exposed chorioallantoic membrane was then flooded with a 1 per cent solution of osmium tetroxide buffered at a pH of 7.6. Portions of the membrane exhibiting the characteristic plaques were cut out with fine scissors, trimmed into cubes of about 1 mm., and fixed at room temperature for 30 minutes in a 1 per cent solution of osmium tetroxide buffered at a pH of 7.6.[19] After washing in three changes of distilled water at 0 C., the specimens were dehydrated in ethyl alcohol of graded dilutions (20, 35, 70, 95%) at 0 C., 20 minutes in each, and in absolute ethyl alcohol, with three changes, for one hour. The tissues were then infiltrated with monomeric methacrylate (4:1 n-butyl and methyl) for two hours and embedded in prepolymerized methacrylate. The process of polymerization was accomplished under ultraviolet light at 30 C. in 24 to 36 hours. The specimens were sectioned by means of a Porter-Blum microtome and examined with an RCA, EMU 3c electron microscope. For control observations, the chorioallantoic membranes of a few apparently normal 14-day chicken embryos were processed by the same technique.

For a comparative light microscopic study of cellular changes encountered in the affected membranes, thick (2 μ) sections were cut from methacrylate-embedded specimens and mounted on glass slides. After removal of methacrylate with chloroform and bleaching in a 3 per cent solution of hydrogen peroxide in 70 per cent ethyl alcohol,[4] the sections were stained with hematoxylin-eosin and by the Feulgen and periodic acid-Schiff (PAS) methods. In addition, some of the involved and normal control membranes were fixed for 24 hours

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in Zenker's fluid, processed through paraffin embedding technique, and stained by the same methods as used for methacrylate-embedded specimens.

Since the shape of intracellular elements is often distorted by compression due to sectioning, the particles presumed to be virus were also studied in purified, shadow-cast preparations. For that purpose, 7 ml. of affected chorioallantoic membranes, harvested five days after inoculation, were suspended in an equal amount of physiological saline solution and homogenized in a Waring Blendor. The extract was freed of gross tissue particles by low-speed centrifugation (500 g) for 20 minutes. The supernatant fluid was then subjected to 90,000 g for one hour in a 39 swinging-bucket rotor of an ultracentrifuge.* The sediment thus obtained was resuspended in 0.3 ml. of physiological saline solution buffered at a pH of 7.0. After deposition on collodion-coated grids, the preparations were dried in the air and shadow-cast with a chrome-gold alloy at a tangent of approximately 20 degrees.

The presence of the LT virus in the membranes under study was tested by infectivity experiment. For that purpose, several apparently healthy chickens, 6 weeks old, which had been kept in isolation from the day of hatching, were inoculated intranasally with 1 cc. of a suspension of the affected chorioallantoic membranes in nutrient broth. A few days after the first signs of clinical illness, the birds were killed and portions of their tracheas ground and extracted with physiological saline solution. The suspension thus prepared was inoculated on the chorioallantoic membrane of nine-day chicken embryos. Five days after inoculation, the membranes were harvested, fixed in Zenker's fluid, and processed for light microscopic studies as described previously.

OBSERVATIONS

Uninfected Membranes.—Since the fine structure of the chorioallantoic membrane of normal chicken embryos has been previously described,[5,13] it is not proposed to enter upon a detailed presentation of its morphological features. The cells of the entodermal layer, primarily concerned in this work, were elongated or polyhedral in shape and usually formed a single layer separating the mesoderm from the allantoic cavity. Their outer surface, in contact with the allantoic fluid, displayed numerous, slender microvilli. The cytoplasm exhibited mitochondria, the elements of endoplasmic reticulum, large vacuoles often containing finely granular material (possibly precipitate), and dense homogenous spheroidal bodies of varying size and apparently of lipid character. The oval or round nuclei often possessed eccentrically placed nucleoli. No particles reminiscent of those observed in infected membranes and presumed to represent the laryngotracheitis virus were found in cells of the normal chorioallantoic membrane.

Infected Membranes.—Light microscopic studies of the affected chorioallantois revealed that the plaquelike lesions were characterized by acute inflammation of the mesodermal layer and by hyperplasia, usually followed by degeneration and necrosis, of the entoderm and only to a minor degree of the ectodermal lining. The appearance of intranuclear inclusion bodies in epithelial cells was a consistent and characteristic feature of the reactive process. The affected nuclei were often enlarged and showed margination of chromatin on the inner surface of the nuclear membrane. On occasion, presumably in earlier stages of formation, the inclusions occupied the entire nuclear space and tended to stain with basic dyes. In many instances, however, the body of the inclusion was separated from the nuclear membrane by a clear zone. A large number of such structures displayed a degree of affinity for acid stains. A small proportion of inclusions showed a strong reaction to the Feulgen test; the remaining ones exhibited only a slight response or were unstained. The PAS technique gave consistently negative results.

In addition to nuclear inclusions, many entodermal cells contained well-defined, intracytoplasmic masses of a finely granular character. The constituent particles were so small that the individual granules could be discerned with difficulty under oil immersion (x 1125). In hematoxylin-eosin stained sections, the aggregates of such particles showed only a moderate affinity for eosin.

A much better differentiation from the cytoplasmic matrix was obtained with the PAS method. A faintly positive reaction could be detected in some of the aggregates with the Feulgen test. These intracytoplasmic "inclusions" varied in size from small round bodies of 1 μ, or less, to large masses of 20 μ in diameter (fig. 1), often displacing the nucleus towards the periphery of the cell. The nature of the aggregates could not be ascertained from examination of stained sections alone. By correlation with the electron microscopic findings, however, it became apparent that most, if not all, of these structures corresponded in

* Spinco Model L ultracentrifuge, manufactured by Beckman Instruments, Inc., Spinco Division, Belmont, Calif.

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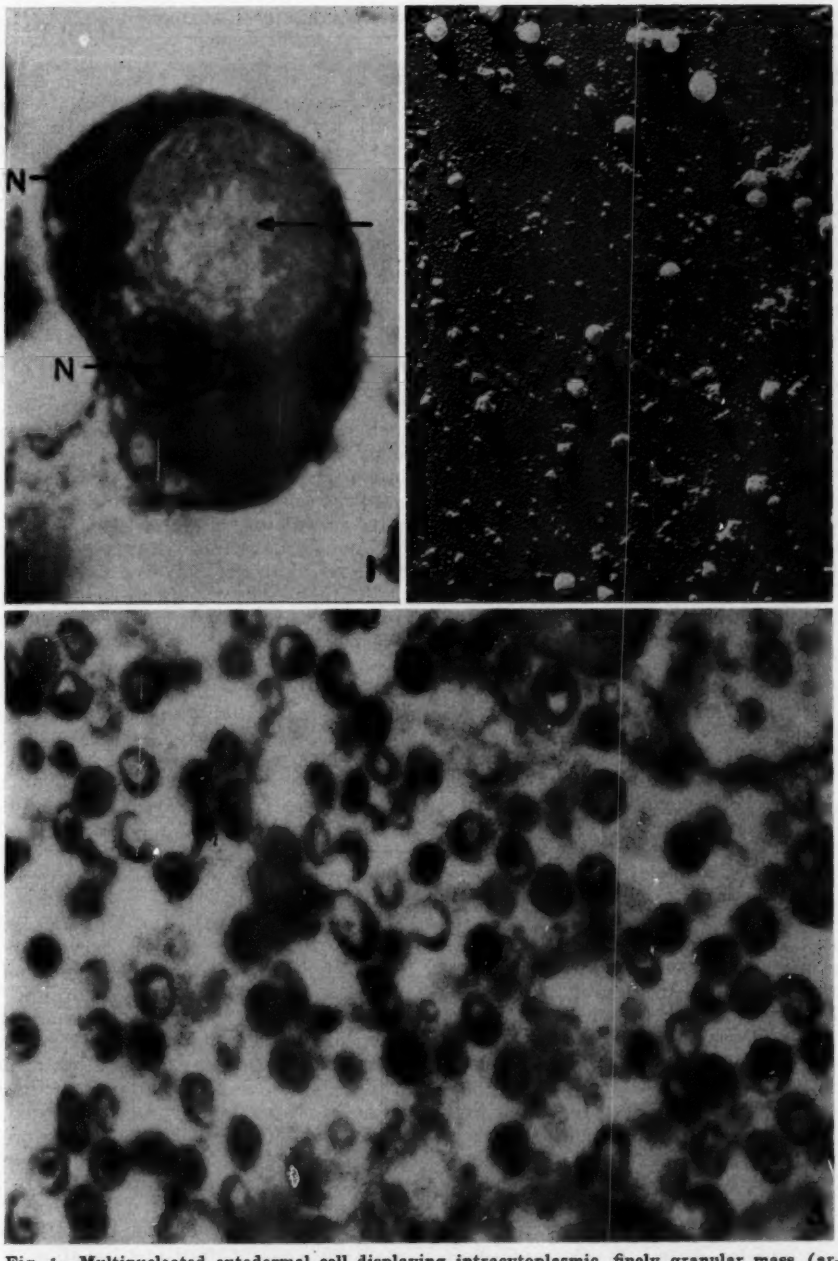

Fig. 1—Multinucleated entodermal cell displaying intracytoplasmic, finely granular mass (arrow) presumed to be composed of laryngotracheitis virus particles. Nuclei (N), showing inclusion bodies in various stages of formation, are displaced toward the periphery. H & E stain; x 2,200.

Fig. 2—Shadow-cast preparation of purified laryngotracheitis virus. Some of the particles of virus are indicated by arrows. x 20,000.

Fig. 3—Electron micrograph of laryngotracheitis virus particles in cytoplasmic aggregate. Most of the particles exhibit an opaque core surrounded by a zone of lesser density and a thick outer membrane. x 38.000.

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their location and size to the masses of particles presumed to be virus.

Electron microscopic observations were focused chiefly on the layer of the chorioallantoic membrane which showed the most characteristic reaction, *i.e.*, the entoderm. Many cells of this layer displayed varying degrees of degeneration. Cytoplasmic changes were manifested essentially by the appearance of vacuoles in the matrix, by swelling and disintegration of the mitochondrial cristae, and by fragmentation of fine tubules which evidently represented the endoplasmic reticulum. Vacuolation of mitochondria was also commonly observed. The cell membrane was sometimes found to be ruptured, seemingly as a result of pressure exerted by marginal vacuoles. The cytoplasmic degenerative processes were often attended by some increase in the number and thickness of the microvilli. Nuclear alterations frequently occurred but were less clearly defined. The most common was the peripheral accumulation of finely granular components which evidently corresponded to margination of chromatin. Rarely, dense and somewhat shrunken nuclei, apparently representing the pyknotic forms, could also be observed.

The degenerative changes in entodermal cells were not infrequently concurrent with the appearance of variable-sized aggregates of well-defined particles presumed to be the virus of infectious laryngotracheitis (fig. 4). In most instances, such particles were present only in the cytoplasm but on rare occasion could also be discerned in the nuclei.

The intracytoplasmic aggregates ranged from small clusters to large accumulations of closely packed particles which occupied a considerable portion of the cell body and displaced the nucleus toward the periphery. Most of the aggregates were enveloped by a thin membrane which often formed a well-delineated vesicle. The number of cells containing such particles varied appreciably from specimen to specimen. On the average, however, approximately 5 per cent of the entodermal cells showed their presence.

The particles found in the cytoplasm measured from 150 mμ to 240 mμ in diameter (fig. 3), with the average size estimated at 180 mμ. Most of the particles displayed a dense core 50 to 80 mμ in diameter, an intermediate zone 40 mμ wide, and an outer membrane which formed an apparently homogenous, dense capsule, measuring 25 to 50 mμ in thickness (fig. 3). The elliptical forms seen in many of the specimens were evidently the result of compression during sectioning. Shadow-cast preparations of the purified virus revealed that the particles were spherical or polyhedral and of 180 mμ in average diameter (fig. 2).

In addition to the viruslike particles, some of the cytoplasmic aggregates contained numerous, fine, smooth-surfaced tubules of varying length and 25 to 45 mμ in width (fig. 7, 8, 9). The tubular character of these structures was evident on cross section (fig. 8). Not infrequently, such tubules displayed a bulbous enlargement at one end (fig. 8), or were apparently terminated by a densely outlined body, resembling in size and shape the outer capsule of the particles (fig. 7). Occasionally, branching forms of uniform width could also be observed (fig. 7).

The intranuclear particles varied appreciably in size and morphological properties. The small forms were moderately dense, homogenous, and of 35 to 40 mμ in diameter. Most of them occurred in small clusters in proximity to the nuclear membrane (fig. 6). The large particles measured 80 to 100 mμ and were often found at the periphery of the nucleus (fig. 5, 6). Their internal structure, however, was more complex and consisted of a dense or ringlike core, 60 mμ in diameter, and an outer zone of lesser density delimited by a very thin membrane (fig. 5).

Infectivity Experiment.—Only mild signs of respiratory distress were observed in the chickens inoculated with a suspension of the affected chorioallantoic membranes. The lesions found on postmortem examination were confined to the trachea and consisted of a patchy hyperemia of the mucous membrane. The extract prepared from the tracheas of these birds and inoculated on the chorioallantoic membrane of nine-day chicken embryos produced a marked reaction in the form of large plaques. On histopathological examination, the involved areas showed inflammatory and hyperplastic changes, accompanied by the appearance of intranuclear inclusion bodies in entodermal cells. These alterations were characteristic of avian infectious laryngotracheitis.

DISCUSSION

The particles described in this report are considered to represent the virus of avian

Fig. 4—Electron micrograph of entodermal cell with one large and few small aggregates of virus particles. No virus is visible in the nucleus (N). Cytoplasm (C) shows numerous, small and irregularly outlined vacuoles (V) representing degenerative changes. x 13,500.

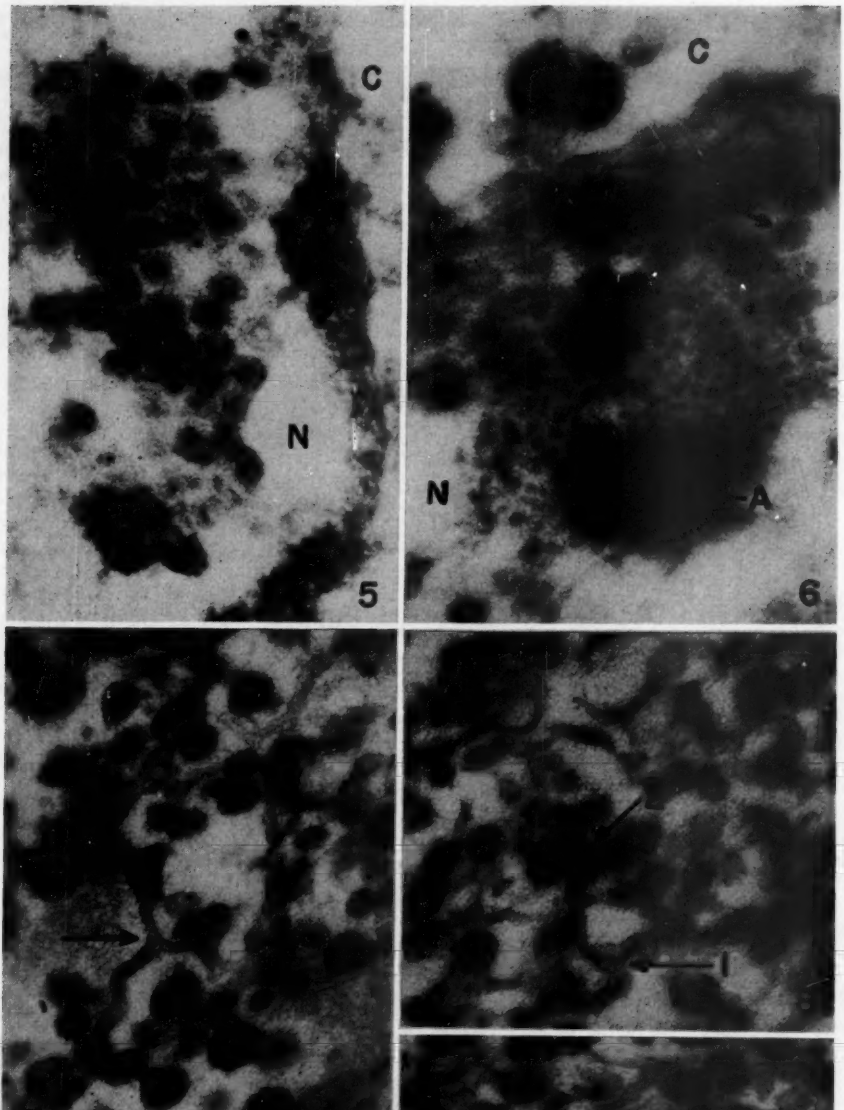

Fig. 5—Intranuclear cluster of virus particles displaying a dense core and a thin outer membrane. N = nucleus; C = cytoplasm. x 60,000.

Fig. 6—Intranuclear cluster of small particles presumed to be of viral nature. Larger forms of virus (arrow), similar to those depicted in figure 5, are also present in proximity to nuclear membrane. Two encapsulated particles are shown in cytoplasm (C). N = nucleus; A = artifact. x 60,000.

Fig. 7–9—Tubular profiles are seen scattered among viral particles in cytoplasmic aggregate. Some of the tubules are branching (fig. 7; arrow), enlarged at one end (fig. 8; arrow-1), or quite long (fig. 9). The small, densely outlined, ovoid structure shown in figure 8 (arrow-2) apparently represents tubule on cross section. x 45,000.

infectious laryngotracheitis because of: (1) their presence only in the infected membranes, (2) consistency of their internal structure, and (3) morphological similarity to some of the viruses, particularly those of the herpes group [17,18,24] and varicella. [28] The viral nature of the particles studied is further substantiated by the results of infectivity tests which demonstrated the presence of the LT agent in the membranes under investigation.

The structure of the cytoplasmic forms of the laryngotracheitis virus closely resembles that of the herpes simplex,[17,18] herpes B,[24] and varicella.[28] All of these viruses, although somewhat smaller than the LT particles, display a dense core, a mid-zone of lesser density, and an outer, double membrane or a capsule. A degree of dimensional and structural similarity is exhibited also by the intranuclear forms, the larger of which reveals a dense core bounded by a zone of lesser density and a single, delimiting membrane. The intranuclear particles of the herpes simplex and varicella have been shown to represent an early stage in the multiplication of virus. From analogy of the structure, it would seem likely that the same situation may exist in respect to the laryngotracheitis virus particles.

Of considerable interest is the appearance of fine, smooth-surfaced tubules within the cytoplasmic aggregates of the LT virus. Such tubules were observed in only a small proportion of affected cells and, as shown (fig. 7, 8, 9), were often associated with the capsule of viral particles. In some morphological aspects, these structures are reminiscent of the smooth-surfaced elements of the endoplasmic reticulum [20,21] and particularly of the membranous profiles of the Golgi complex.[9,10,22]

Although the origin of the tubules is at present unknown, it seems entirely possible that, under conditions of cellular degeneration induced by virus, a disorganization of the Golgi system could result in the appearance of the morphologically similar structures. In this connection, it is of interest that both the Golgi complex [8,9,14,25] and the aggregates of virus, in which the tubules are found, give in many instances a positive PAS reaction.

Close relationship of virus particles with tubular or filamentous, intracytoplasmic structures has been previously reported. Bernhard *et al.*[3] observed thin lamellar profiles, arranged in parallel fashion, in association with particles of virus in Shope fibroma and tentatively interpreted them as by-products of virus metabolism. Smooth-surfaced tubules scattered among densely packed particles of presumably viral character were found by Selby *et al.*[27] in areas corresponding to Golgi zones of Ehrlich ascites tumor cells. Friedlaender *et al.*[12] showed, in their studies on the growth of Anopheles A virus in tumor cells, that viral particles were harbored and evidently propagated within the rough-surfaced elements of the endoplasmic reticulum. Recently, Fawcett [11] demonstrated dense, 25-mμ thick filaments and occasional tubular profiles in close relationship to virus particles of Lucké renal adenocarcinoma.

At present, no satisfactory interpretation can be offered concerning the origin and significance of the tubular structures described in this report. Further studies, now in progress, should provide more information on the origin of the tubules and on the development of the laryngotracheitis virus.

SUMMARY

The structure of the virus of avian infectious laryngotracheitis was studied in sections of the chorioallantoic membranes of chicken embryos. Five days after inoculation of nine-day embryonating chicken eggs, the membranes were harvested, fixed in osmium tetroxide buffered at a pH of 7.6 and, following dehydration in ethyl alcohol of graded dilutions, embedded in methacrylate.

Viral particles were seen most commonly in the cytoplasm and rarely within the nuclei of entodermal cells. The intracytoplasmic forms ranged from 150 to 240 mμ in diameter and displayed an opaque core measuring 50 to 80 mμ, a mid-zone of lesser density, and an outer capsule, 25 to 50 mμ thick. The intranuclear particles of virus varied in size from 30 to 40 and from 80 to 100 mμ in diameter, with only the larger forms possessing an outer coat delineated by a very thin membrane. Occasionally, slender and sometimes branching tubules occurred among the viral particles of cytoplasmic aggregates. A number of such tubules appeared to be closely associated with the capsules of virus.

References

[1] Beach, J. R.: The Virus of Laryngotracheitis of Fowls. Science, 72, (1930): 633–634.

XUM

[2] Beach, J. R.: A Filterable Virus, the Cause of Infectious Laryngotracheitis of Chickens. J. Exptl. Med., *54*, (1931): 809–816.

[3] Bernhard, W., Bauer, A., Harel, J., and Oberling, C.: Les formes intracytoplasmatiques du virus fibromateux de Shope. Études de coupes ultrafines au microscope électronique. Bull. A. franç. étude cancer, *41*, (1954): 423–444.

[4] Bloch, D. P., Morgan, C., Godman, G. C., Howe, C., and Rose, H. M.: A Correlated Histochemical and Electron Microscopic Study of the Intranuclear Crystalline Aggregates of Adenovirus (RI-APC virus) in HeLa Cells. J. Biophys. & Biochem. Cytol., *3*, (1957): 1-8.

[5] Borysko, E., and Bang, F. B.: The Fine Structure of the Chorio-Allantoic Membrane of the Normal Chick Embryo. Bull. Johns Hopkins Hosp., *92*, (1953): 257-289.

[6] Brandly, C. A.: Some Studies of Infectious Laryngotracheitis. The Continued Propagation of the Virus Upon the Chorio-Allantoic Membrane of the Hen's Egg. J. Infect. Dis., *57*, (1935): 201–206.

[7] Burnet, F. M.: The Propagation of the Virus of Infectious Laryngotracheitis on the Chorio-Allantoic Membrane of the Developing Egg. Brit. J. Exptl. Path., *15*, (1934): 52–55.

[8] Chu, C. H. U., and Swinyard, C. A.: Morphological and Cytochemical Identification of the Golgi Apparatus. J. Biophys. & Biochem. Cytol., *2*, (1956): 263–280.

[9] Dalton, A. J., and Felix, M. D.: Cytologic and Cytochemical Characteristic of the Golgi Substance of Epithelial Cells of the Epididymis—*in Situ* in Homogenates and After Isolation. Am. J. Anat., *94*, (1954): 171–207.

[10] Dalton, A. J., and Felix, M. D.: A Comparative Study of the Golgi Complex. J. Biophys. & Biochem. Cytol., *2*, (Suppl.), (1956): 79–83.

[11] Fawcett, D. W.: Electron Microscope Observations on Intracellular Virus-Like Particles Associated with the Cells of the Lucké Renal Adenocarcinoma. J. Biophys. & Biochem. Cytol., *2*, (1956): 725–741.

[12] Friedlaender, M., Moore, D. H., and Koprowski, H.: Studies with the Electron Microscope of Virus-Host Relationships in Ehrlich Ascites Tumor Cells. II. The Localization and Possible Development of Anopheles A Virus Within the Endoplasmic Reticulum of the Host Cell. J. Exptl. Med., *102*, (1955): 371–377.

[13] Gaylord, W. H., Jr., and Melnick, J. L.: Intracellular Forms of Pox Viruses as Shown by the Electron Microscope (Vaccinia, Ectromelia, Molluscum Contagiosum). J. Exptl. Med., *98*, (1953): 157–171.

[14] Gersh, I.: A Protein Component of the Golgi Apparatus. Arch. Path., *47*, (1949): 99–109.

[15] Gibbs, C. S.: Ultrafiltration Experiments with the Viruses of Laryngotracheitis and Coryza of Chickens. J. Bact., *30*, (1935): 411–417.

[16] Gorham, J. R.: A Simple Technique for the Inoculation of the Chorioallantoic Membrane of Chicken Embryos. Am. J. Vet. Res., *18*, (July, 1957): 691–692.

[17] Morgan, C., Ellison, S. A., Rose, H. M., and Moore, D. H.: Internal Structure in Virus Particles. Nature (London), *173*, (1954): 208.

[18] Morgan, C., Solon, A. E., Rose, H. M., and Moore, D. H.: Structure and Development of Viruses as Observed in the Electron Microscope. I. Herpes Simplex Virus. J. Exptl. Med., *100*, (1954): 195–202.

[19] Palade, G. E.: A Study of Fixation for Electron Microscopy. J. Exptl. Med., *95*, (1952): 285–298.

[20] Palade, G. E.: Studies on the Endoplasmic Reticulum. II. Simple Dispositions in Cells *in Situ*. J. Biophys. & Biochem. Cytol., *1*, (1955): 567–582.

[21] Palade, G. E.: The Endoplasmic Reticulum. J. Biophys. & Biochem. Cytol., *2*, (1956): 85–97 [Suppl.].

[22] Palay, S. L., and Palade, G. E.: The Fine Structure of Neurons. J. Biophys. & Biochem. Cytol., *1*, (1955): 69–88.

[23] Reagan, L. R., Delaha, E. C., Cook, S. R., Brueckner, A. L., Porter, J. E., and Moulthrop, I. M.: Electron Microscopic Studies of Laryngotracheitis Virus. Am. J. Vet. Res., *16*, (Jan., 1955): 147–148.

[24] Reissig, M., and Melnick, J. L.: The Cellular Changes Produced in Tissue Cultures by Herpes B Virus Correlated with the Concurrent Multiplication of the Virus. J. Exptl. Med., *101*, (1955): 341–351.

[25] Schneider, W. C., and Kuff, E. L.: On the Isolation and Some Biochemical Properties of the Golgi Substance. Am. J. Anat., *94*, (1954): 209–224.

[26] Seifried, O.: Histopathology of Infectious Laryngotracheitis in Chickens. J. Exptl. Med., *54*, (1931): 817–825.

[27] Selby, C. C., Grey, S., Lichtenberg, S., Friend, C., Moore, A. E., and Biesele, J. J.: Submicroscopic Cytoplasmic Particles Occasionally Found in the Ehrlich Mouse Ascites Tumor. Cancer Res., *14*, (1954): 790–794.

[28] Tournier, P., Cathala, F., and Bernhard, W.: Ultrastructure et développement intracellulaire du virus de la varicelle observé au microscope électronique. Presse méd., *65*, (1957): 1229–1234.

Newcastle Disease Virus in the Aqueous Humor of Chickens

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CLARK *et al.*[3] reported the use of the aqueous humor from eyes of chickens in the incipient stages of Newcastle disease (ND) as a source of antigen for hemagglutination (HA) testing. They indicate that the virus exists in this fluid before the blood serum inhibits the HA reaction. From further studies with birds that did not show signs of ND, these authors [4] concluded that the aqueous humor is a reservoir for Newcastle disease virus (NDV) in a large percentage of chickens in southern California.

The purpose of this study was to determine whether NDV could be isolated from chickens experimentally vaccinated and challenged and if NDV could be isolated from chickens with clinical and subclinical field cases.

MATERIALS AND METHODS

A total of 127 Rhode Island Red chickens, 18 to 20 weeks old, from the University of Rhode Island poultry flock, was used in this trial. Ten of the birds which were neither vaccinated nor challenged served as controls (table 1). The rest were given one or two doses of various types of killed and live ND vaccine when 2 to 6 weeks old. At 18, 20, or 22 weeks of age, the immunity of each experimental chicken was challenged with 2.5×10^6 e.l.d.$_{50}$, intratracheally, of the BG strain of NDV.[2]

TABLE 1—Results of Tests of the Aqueous Humor of Newcastle Disease-Vaccinated Birds Following Challenge

No. birds	Age at challenge (weeks)	Time from challenge to collection of samples (weeks)	Virus isolation	HI titer of samples
46	18	3	0	...
37	20	3	0	...
5	Controls	...	0	...
34	22	3	...	8–64
5	Controls	...	...	0

HI = hemagglutination inhibition; ... = neither virus isolation or HI testing was carried out.

Three weeks after the immunity of the vaccinated birds had been challenged, all were killed. The aqueous humor was collected from both eyes of each bird and pooled. The method of collection was that described by Clark *et al.*[3] The specimens were frozen immediately and stored at −20 C. until tested. Before inoculation, the samples of aqueous humor were mixed with 100 mg. of streptomycin per milliliter, and were left at room temperature for 15 minutes. Specimens from 88 chickens, including 5 controls, were inoculated into 10-day-old chicken embryos in groups of 5. Each embryo was given 0.15 ml. of the fluid mixture in the chorioallantoic chamber. The eggs were candled twice daily during incubation until hatching time.

Thirty-nine samples, including five from the control birds (table 1), were tested for hemagglutination-inhibition (HI) antibodies. The serums were diluted 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 for *beta* HI procedure.

Virus isolations also were attempted from aqueous humors of 18 groups of chickens. These were birds with field cases which were submitted to the laboratory for diagnosis. Of these birds, those in five groups showed respiratory or nervous signs, or both. Birds in two groups showed only nervous signs. The rest of the chickens did not show signs of ND, but had been vaccinated for the disease.

RESULTS

As indicated (table 1), the attempts to isolate NDV from 83 vaccinated and challenged birds, as well as from 5 control birds, were unsuccesful. The HI test conducted on the aqueous humor samples from the remaining 34 vaccinated chickens showed HI titers of 8 to 64. The samples from the 5 control birds were negative.

Of the chickens that were submitted to the laboratory for diagnosis, NDV was isolated from the aqueous humors of birds in five groups that were showing respiratory or nervous signs, or both (table 2). The virus was not recovered from birds in the two groups showing nervous signs only. The virus also was not isolated from the 11 groups which had been vaccinated and that did not show nervous or respiratory signs.

DISCUSSION

Clark *et al.*[4] tested the aqueous humors of 234 groups of chickens vaccinated with different types of ND vaccines; 191 groups

Contribution No. 946, from the Department of Animal Pathology, Agricultural Experimental Station, University of Rhode Island, Kingston.

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TABLE 2—Histories of Birds with Field Cases and Results of Virus Isolations from Aqueous Humors

No. birds with field cases	No. birds examined	Clinical signs	Approximate time of examination after onset of disease	Newcastle disease vaccination history	Newcastle disease virus isolation
5	17	Nervous, respiratory or both	5 days	No	Yes
2	11	Nervous signs only	10–14 days	No	No
11	42	No overt respiratory signs	.....	Yes	No

were HI positive. They also reported the isolation of the virus from the aqueous humors of 111 of 127 groups of chickens, whose histories and signs were not suggestive of ND.

Zuijdam [7] found that flocks of chickens partially immune as a result of vaccination with a killed or live ND vaccine excreted the virus for 33 and 19 days after challenge with virulent virus. He also stated that the virus was not shed when birds were vaccinated with two other live ND vaccines and subsequently challenged.

In the experiments, it was not possible to isolate NDV from the aqueous humors of birds vaccinated with live or killed vaccines or both when 2 to 6 weeks old and challenged intratracheally when 18 to 22 weeks old. However, HI antibodies were demonstrated in the aqueous humors of vaccinated chickens that survived challenge.

Newcastle disease virus has been shown by Karzan and Bang [5] to be present in the tracheal exudate, brain, spleen, blood, and lungs. Sinha *et al.*[6] also isolated it from the muscles during the early stages of the disease. Much difficulty may be experienced in demonstrating the virus in tissues of birds with cases of a few days' duration, according to Beach.[1]

In this trial, NDV was isolated from the aqueous humor of the eyes, as well as from the tracheal fluids, of the birds that were showing respiratory and nervous signs. It was not isolated from birds that were showing nervous signs only.

It is to be expected that, at some time during the appearance of clinical signs, NDV would be circulating in the body of the chicken and may be found in the aqueous humor. Further investigation is needed to determine the persistence of the virus in the aqueous humor of convalescent and immune birds.

Under the conditions of this experiment, it appears that the aqueous humor is not a reservoir for NDV. If the virus persists in some organ of the chicken without clinical signs being manifested, the problem of determining how widely the disease is distributed becomes difficult, since a successful program of control and eradication of the disease should take the carrier bird, if any, into consideration.

SUMMARY

1) Attempts to isolate Newcastle disease virus from the aqueous humors of 127 vaccinated birds that survived challenge failed; however, hemagglutination-inhibition antibodies were present in the 34 birds tested.

2) Newcastle disease virus was isolated from the tracheas and aqueous humors of naturally infected chickens showing respiratory signs of Newcastle disease.

3) The aqueous humor of chickens was not shown to be a reservoir for Newcastle disease virus.

References

[1] Beach, J. R.: Diseases of Poultry. 2nd ed. Iowa State College Press, Ames (1948): 492.

[2] Boney, W. A.: The Isolation of Neurotropic Strain (G.B.) of Newcastle Disease Virus. Southwest. Vet., *5*, (1951): 19–21.

[3] Clark, D. S., Jones, E. E., and Ross, F. K.: The Use of Aqueous Humor for Early Diagnosis of Newcastle Disease. Am. J. Vet. Res., *16*, (Jan., 1955): 138–140.

[4] Clark, D. S., Jones, E. E., and Ross, F. K.: Further Studies with the Aqueous Humor of Chickens as a Reservoir for the Virus of Newcastle Disease. Am. J. Vet. Res., *18*, (Jan., 1957): 204–206.

[5] Karzan, D. T., and Bang, F. B.: The Pathogenesis of Infection with a Virulent (B) Strain of Newcastle Disease Virus in the Chicken. J. Exptl. Med., *93*, (1951): 267–284.

[6] Sinha, S. K., Hanson, R. P., and Brandly, C. A.: Comparison of the Tropism of Six Strains of Newcastle Disease Virus in Chickens Following Aerosol Infection. J. Infect. Dis., *91*, (1952): 276–282.

[7] Zuijdam, D. M.: Diseases of Poultry. 3rd ed. Iowa State College Press, Ames (1952): 550.

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The Response of the Developing Chicken Embryo to Certain Avian Pathogens

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THE EARLY WORK of Woodruff and Goodpasture[32] initiated widespread use of chicken embryos for the cultivation of pathogens. The gross pathology, embryo mortality, and the condition of the extrafetal membranes of experimentally infected embryos have been reported extensively; however, there is a paucity of histopathological studies. Microscopic studies of embryos infected with rabies virus,[7,21,27] poliomyelitis virus,[24,25] infectious bronchitis virus,[23] and avian pleuropneumonia-like organisms (PPLO)[2,31] have been made. The present investigation is concerned with the pathological response of the embryo to avian encephalomyelitis (AE) virus, infectious synovitis virus, avian infectious hepatitis (AIH) agent, avian pleuropneumonia-like organism, Newcastle disease (ND) virus, and infectious bronchitis (IB) virus.

MATERIALS AND METHODS

In studies of AE, the inoculum consisted of ground brain, prepared by the method of Feibel *et al.*,[9] from AE-infected chicks. All other inoculums consisted of yolk material harvested from infected embryos. One tenth milliliter of the first tenfold serial dilution was inoculated into embryos. The broth-cultivated PPLO strains were used undiluted in the form of 24-hour agifated Bacto PPLO Broth* cultures, without crystal violet, but enriched with 1 per cent PPLO Serum Fraction.* All control embryos were inoculated with 0.1 ml. of the diluent. The AIH agent was inoculated into the amnioallantoic cavity of 10-day embryos, and other agents were inoculated into the yolk sac of 6- to 7-day embryos. A PPLO-negative flock supplied the 2,500 embryonating eggs of uniform size employed in this study.

At postinoculation (PI) day 2 to 3 and continuing to incubation day 18 to 19, 1 to 3 infected live embryos and an equal number of diluent-inoculated control embryos were removed daily for examination. The embryos were thoroughly exsanguinated by severing the umbilical vessels or by decapitation. Embryos were weighed, and gross external and internal changes recorded, if present. The embryos were fixed in Bouin's solution. After fixation, eight cross-sectional levels through the embryo and one longitudinal section through the thigh were made. The blocks were embedded in paraffin and sectioned at 10 μ. Sections were routinely stained with Harris' hematoxylin and aqueous eosin. Special staining techniques were employed where indicated.

RESULTS

AVIAN ENCEPHALOMYELITIS

Gross Observations.—Beginning on PI day 8 (incubation day 15) and continuing to the end of the observation period, all infected embryos were inactive, reduced in size, and had stiff limbs with twisted and abnormally placed toes. Muscle atrophy was severe and undoubtedly contributed to the statistically significant weight difference between infected and control embryos. At PI day 13 (incubation day 20), the infected embryos weighed an average of 12.68 Gm. each as opposed to 22.08 Gm. for the controls. Cross sections of brains in the terminal incubation period revealed a severe internal hydrocephalus (fig. 1, 2). Although extensive gross and microscopic changes developed by the second trimester of incubation, no embryos died before incubation day 19.

Microscopic Observations.—Spinal Cord.—The first lesion produced was observed at PI day 5 (incubation day 12) in the large ventral motor neurons of the ventral horn. The cells became swollen and vacuolated, showing peripheral displacement of the

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* Produced by Difco Laboratories, Detroit, Mich.

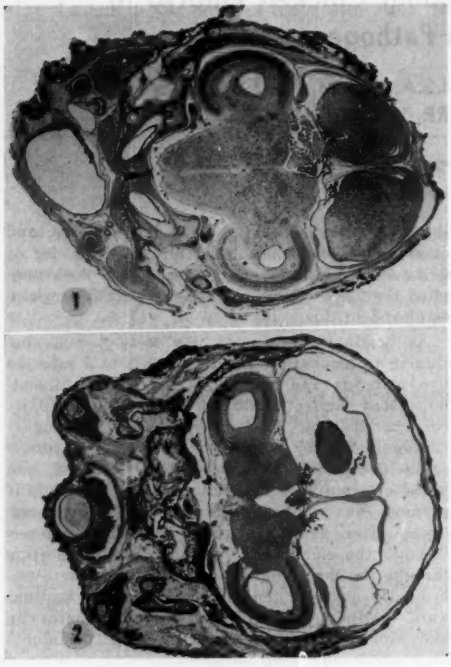

Fig. 1—Cross section of brain of 18-day-old control embryo injected with diluent. H & E stain; x 4.

Fig. 2—Cross section of brain of 18-day-old embryo infected with avian encephalomyelitis. There is hydrocephalus and decrease in nervous tissue. H & E stain; x 4.

nucleus or axonal reaction. With further incubation, the degeneration extended to the dorsal horn. The damaged neurons disappeared rapidly but without inciting an inflammatory response. Thus, at PI day 7 (incubation day 14) the size of the cord was markedly reduced, the space ordinarily occupied by the spinal cord being devoid of tissue other than fibrous tags passing from the meninges to the atrophied cord (fig. 3, 4).

The meninges appeared slightly edematous and the subarachnoid space distended with fluid. From PI day 11 to 13 (incubation day 18 to 20), the cord changes became profound; little functional spinal cord persisted. The ependymal cells appeared normal at all times.

Dorsal Ganglia.—At PI day 6 (incubation day 13), the dorsal ganglia of infected embryos showed numerous hyperchromatic neurons with pyknotic or karyorrhectic nuclei. With additional incubation, these changes were accompanied by scattered infiltration of heterophils. In the terminal incubation period, the ganglia consisted of a few neurons and of fibrous tissue, thus reflecting the changes which occurred in the spinal cord. The closely associated sympathetic ganglia were not involved.

Brain.—The first changes in the brain were noted at PI day 7 (incubation day 14). The lesions were essentially those occurring earlier in the cord. They differed in that both diffuse and occasionally circumscribed

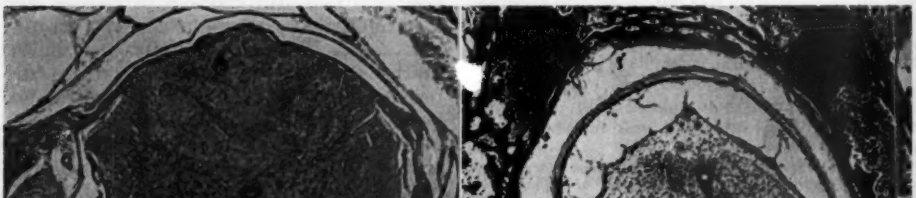

Fig. 3—Cross section of cervical cord of 14-day-old control embryo injected with diluent. H & E stain; x 34.

Fig. 4—Cross section of cervical cord of 14-day-old embryo infected with avian encephalomyelitis. There is dilatation of pia-arachnoid space and atrophy of cord. H & E stain.

heterophilic infiltrations occurred. Neuronophagia, gliosis, and satellitosis were not prominent features. There was no evidence of ischemic necrosis, glial damage, demyelination, or ependymal injury. In later stages of development, areas of encephalomalacia occurred in the midbrain and cerebrum. At PI day 12 to 13 (incubation day 19 to 20), the cerebral hemispheres, optic lobes, and medulla oblongata were reduced to mere shells, consisting of glial cells and small neurons, thus substantiating the gross observation of a severe internal hydrocephalus.

Skeletal Musculature.—It was difficult to evaluate changes in embryonic skeletal muscle at incubation day 11 to 13 because nuclei were numerous, fiber formation was just commencing,[17] and degenerative changes (a normal developmental phenomenon)[1,11] were present, accompanied by actively dividing cells. At incubation day 18 to 20, the muscle changes were so pronounced that no doubt existed as to their pathological nature. Thus it remained to evaluate early changes and to determine their onset. At PI day 5 (incubation day 12), a few bundles were swollen and exhibited pyknotic, centrally located nuclei and hyperchromatic peripheral myofibrillae. The myopathy progressed rapidly so that, terminally, there was little functional skeletal muscle present in the body. The muscle remnants consisted of ill-defined shrunken bundles with pyknotic nuclei and short, indistinct fibers surrounded by edematous connective tissue.

Articulations.—Bones appeared to undergo normal chondrification and ossification. By the final trimester of incubation, the femorotibial articulation was frequently fused by a dense connective tissue. Other articulations were not examined.

Pathogenesis.—Avian encephalomyelitis virus elicited responses in the chicken embryo[20] similar to those described by Love and Roca-Garcia[24,25] for poliomyelitis virus. Both acted upon the large motor neurons of the ventral horns. The AE virus varied in that it caused a much more advanced condition, deleting the ventral horns and white matter and reducing the dorsal horns; in the brain, it produced an encephalomalacia accompanied by a hydrocephalus. Poliomyelitis virus did not elicit a heterophilic reaction in the brain as did the AE virus. The myopathy of AE-infected embryos was much more profound than in polio-infected embryos. Love and Roca-Garcia[25] reported that the poliomyelitis virus introduced into the yolk sac multiplied there and initiated a viremia. From the blood, the virus entered directly into the nervous system at widely scattered points where it multiplied and produced lesions.

In experimental embryology employing limb bud techniques,[12,15] the differentiation and development of the skeletal muscles was found dependent upon the degree of innervation of the limb. If it was not innervated, the musculature was markedly impaired and degeneration and atrophy occurred. In consequence, the dorsal ganglia, the size of which varied with the peripheral muscle load, became degenerated and atrophied. It remains to be verified that a similar pathogenesis occurs in AE-infected embryos. However, the order of appearance of lesions would suggest that the virus attacked the central nervous system and initiated secondary muscular dystrophy and dorsal ganglionic atrophy. The inactivity of limbs would contribute to the ankylosis, similar to that occurring in nerveless limb transplants.[15]

The embryonic response to AE virus varied intrinsically from that in postembryonic life. In the latter, AE is characterized by proliferative rather than degenerative changes. Neurogliar and lymphocytic infiltrations occur around the capillaries of the brain and lymphocytic follicles are found in certain viscera.[19]

INFECTIOUS SYNOVITIS

Gross Observations.—Infected embryos were smaller in size than control embryos, averaging 8.7 Gm. as opposed to 13.7 Gm. at PI day 8 (inoculation day 15). In no instance was arthritis, tendovaginitis or bursitis observed in infected embryos. Hepatomegalia and distinct splenomegaly occurred.

Microscopic Observations.—Liver.—At PI day 5 (incubation day 12), short cords and clumps of hyperchromatic cells occurred free in or abutted the hepatic sinusoids. The cells had dark round or oval nuclei, slightly smaller than the adjacent hepatic cord cell nuclei, and were surrounded by a small amount of granular cytoplasm. The nuclei stained much darker than the hepatic cord cell nuclei and did not have the prominent nucleoli of the latter. Some of the nuclei were elongated and positioned

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along the sinusoidal wall, thus resembling Kupffer's cells and suggesting free cells originating from the vascular endothelium of the sinusoids. Mitotic figures were frequent (fig. 5–8). At PI day 9 (incubation day 16) and continuing throughout the observation period, similar cells occurred around the hepatic blood vessels and beneath Glisson's membrane. These cells were numerous and formed characteristic dark cuffs about the portal and central vessels (fig. 9). When stained with Wilder's reticulum stain, the hyperchromatic cells were found associated with numerous reticular fibers.[18] Trypan blue, when injected into the air sacs of embryonating eggs, using the method of Hanan,[16] was readily phagocytized by the hyperplastic cells. These results indicated that the hyperplastic cells were reticulocytes.

Blood Vessels of Other Organs and Tissues.—Though most prominent in the liver, reticular cells occurred with less frequency around the blood vessels supplying the mesentery, gizzard, skeletal muscles, and leptomeninges. In all instances, the reticular cells were associated with medium-sized to small blood vessels.

Spleen.—It was difficult to assess changes in the spleen; however, it was apparent that the red pulp was composed predominantly of myeloid cells. There was no increase in reticular fibers nor was trypan blue phagocytized.

The described lesion was produced by six different strains of synovitis virus studied.

Pathogenesis.—Sugiyama[30] reported that in chicken embryos, extravascular phagocytes arose from the mesenchyma as early as incubation day 2 and that basophilic leukocytes first occurred at incubation day 14; typical lymphocytes and eosinophils did not appear until the terminal incubation period. Sabin[26] showed that phagocytes could arise intravascularly from the endothelial cells of blood vessels even in postembryonic life. They could also arise extravascularly from mesenchymal cells enclosing and surrounding the adventitia of blood vessels in embryos. Mesenchymal cells have the potential of forming adventitial cells of blood vessels, histiocytes, and myeloblasts.[3–6] Morphology, location, special staining, and the ability to phagocytize trypan blue indicated that the hyperplastic cells were reticular cells derived from the vascular endothelium and mesenchyma surrounding the blood vessels.

The splenomegaly occurring in infected embryos was difficult to interpret. By grafting adult splenic tissue on the allantois of developing embryos, Danchakoff[3–6] produced splenomegaly thought to be the result of mesenchymal cells becoming granulopoietic and converting the spleen into a mass of myeloid cells. It is possible that a similar transformation occurred in synovitis-infected embryos since, aside from myeloid cells, no abnormal cell type was recognized.

The embryo's response to infectious synovitis virus resembles that in the adult. Sevoain *et al.*[28,29] reported that the livers of infected chickens showed perivascular, periportal, and intraparenchymal cellular hyperplasia. The nature of the hyperplastic cells was verified by reticular stains but not by examination for phagocytic properties. Because of their morphology, location, and mitotic activity, these cells were also interpreted as reticular endothelial cells.

Avian Infectious Hepatitis

Gross Observations.—The weights of infected embryos did not vary from those of the controls. The livers were frequently of an intense green color and showed numerous small creamy areas on their surfaces.

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Fig. 5—Cross section at lumbar level of 16-day-old control embryo, injected with diluent: liver (A), duodenum (B), mesonephros (C), metanephros (D), spinal cord (E) and spleen (F). H & E stain; x 8.

Fig. 6—Cross section of 16-day-old embryo infected with synovitis. There is hepatomegalia and splenomegaly. Lettering as in figure 5. H & E stain; x 8.

Fig. 7—Liver of 12-day-old control embryo injected with diluent. Hepatic cord cells and sinusoids are normal. H & E stain; x 730.

Fig. 8—Liver of 12-day-old embryo infected with synovitis. There are numerous reticular cells free in sinusoids. H & E stain; x 730.

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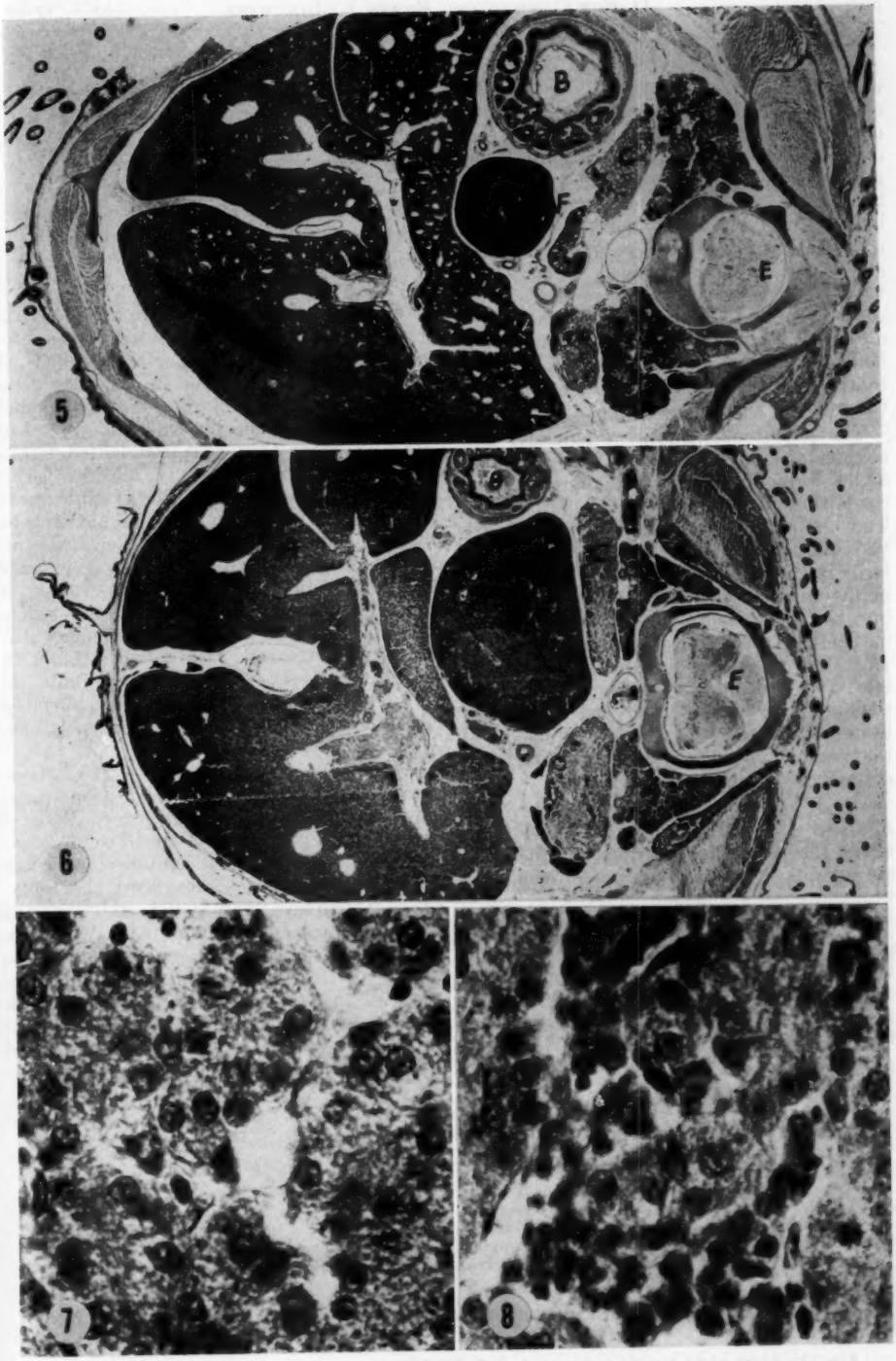

Figures 5–8

Microscopic Observations.—A distinct focal necrosis was characteristic of the livers of infected embryos after PI day 6 (incubation day 16). The cord architecture was disrupted in circumscribed areas, and normal hepatic cells were replaced by necrotic, haphazardly located cells and by cellular debris (fig. 10, 11). Also characteristic were numerous proliferated bile ducts, bile stasis, and occasional areas of scar tissue. Individual hepatic cord cells were swollen and their cytoplasms were pale. Occasionally, a hepatic cord cell would appear dark and homogenous, standing out in sharp contrast to the surrounding cells. Heterophils occurred diffusely in the parenchyma.

The portal vessels were frequently surrounded by large, actively dividing cells which were, on occasion, arranged in short cords that blended with the hepatic cords. The nucleus of the proliferating cell was vesicular, round, and usually exhibited a large nucleolus. The scant cytoplasm had a mauve tinge; thus, not only the nucleus, but the cytoplasm, was more hyperchromatic than the adjacent hepatic cord cells (fig. 12). The use of modified Forsgren's [10] bile duct-staining technique brought out the similarity of the liver cord cells to the hyperplastic cells, in that the nucleoli of the periportal cells and the hepatic cells stained with equal intensity.

Focal necrosis, bile duct proliferation, bile stasis, and liver cord cell hyperplasia about the portal vessels were produced by six strains of AIH agent when inoculated into developing chicken embryos.

Pathogenesis.—The agent of AIH appeared to have a specific necrotizing action upon the hepatic parenchyma. Such necrosis interfered with the hepatic metabolism, resulting in bile stasis and bile duct proliferation. The arrangement of the periportal cells into short cords blending with the hepatic cords, and the similarity of the nuclei and nucleoli of the periportal cells and hepatic cord cells, suggested that these elements were young multiplying liver cord cells.

The response of the chicken embryo to the AIH agent resembled that occurring in postembryonal infections.[29]

AVIAN PLEUROPNEUMONIA-LIKE ORGANISM

Observations.—Liver.—No gross alterations were observed. The earliest microscopic lesion in infected embryos consisted of cellular proliferation in the adventitia of the hepatic vessels at PI day 6 (incubation day 13). The hyperplastic cells had nuclei varying from oval to indented, horseshoe-shaped or completely bilobed forms joined by a fine filament. The granules were so minute that their shape could not be determined in fixed sections. Because typical eosinophils were said to occur only terminally in the incubation period,[30] it was concluded that these cells were immature heterophils. Nuclear morphology differentiated the cells from myelocytes. With continued incubation, the heterophils increased in number and maturity, forming irregular aggregates about the vessels (fig. 12).

Other Organs and Tissues.—The heterophilic perivasculitis was most pronounced in the liver but occurred also in the gizzard musculature, the serosa and, to a lesser degree, in the skeletal muscles and loose connective tissue throughout the body. The mesentery of infected embryos was markedly thickened by a massive heterophilic infiltration apparently related to blood vessels. At PI day 10 to 11 (incubation day 17 to 18), periarteritis developed in the lungs (fig. 13, 14). In sharp contrast to the mature heterophils occurring around the hepatic vessels at this age, the cells sur-

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Fig. 9—Liver of 15-day-old embryo infected with synovitis. There is hyperplasia of reticular cells around the blood vessels and beneath Glisson's capsule. In the black and white photo, these cells appear similar to the heterophils in figure 12. They are recognizable under the microscope by their lack of cytoplasmic eosinophilic granules and other features. H & E stain; x 38.

Fig. 10—Liver of 16-day-old embryo infected with infectious hepatitis. There is focal necrosis. H & E stain; x 150.

Fig. 11—Liver of 16-day-old embryo infected with infectious hepatitis. The portal vein (center) is surrounded by proliferated hepatic cord cells. H & E stain; x 150.

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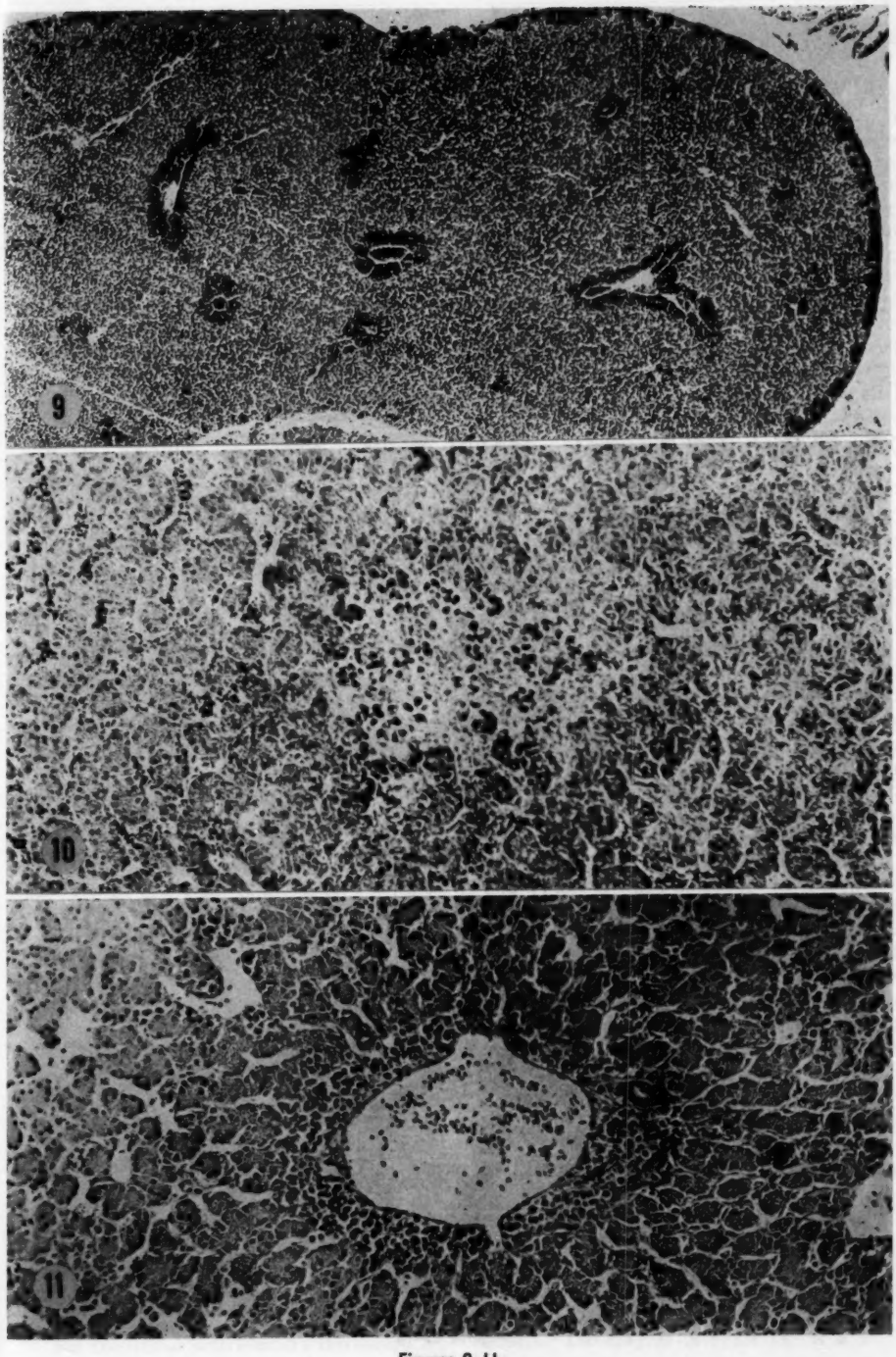

Figures 9–11

Figures 12–14

rounding the arteries of the lung were immature. No other alterations were observed in the lung.

The characteristic heterophilic perivasculitis was produced by ten of 13 strains studied. Included in the ten strains were chicken red blood cell-agglutinating and nonagglutinating strains that produced either rough or smooth colonies on solid mediums. The strains producing rough colonies induced a more intense perivasculitis than those producing smooth colonies. One strain producing perivasculitis had undergone 540 successive broth passages.

Pathogenesis.—The sole lesion produced by avian PPLO in this study was an extensive heterophilic perivasculitis around the small and medium-sized blood vessels, especially those of the liver. These findings are in sharp contrast to the reports of other workers who described a variety of microscopic changes in the chicken embryo.[2,31] Such variation may possibly be ascribed to the study of dead or chill-killed embryos in the earlier studies. Dead embryos have been found to undergo autolysis, become edematous and hemorrhagic as a result of imbibition or agonal diapedesis. The accumulation of blood cells produces a highly cellular state that may mask or simulate pathological changes and increase the already high cellular state of the embryo.

While avain PPLO has a predilection for the respiratory tract in the adult,[5,31] such proclivity was not characteristic in embryos in this study. Van Roekel *et al.*[31] indicated that microscopic lesions in the respiratory tract developed in the terminal incubation period (incubation day 20.9). In the present investigation, avian PPLO elicited a specific periarteritis in the lungs at incubation days 17 and 18, which period was thought to coincide with the normal development of arterial vascularization of this organ.[22]

Newcastle Disease Virus and Infectious Bronchitis Virus

Observations.—No consistent pathological lesions were produced by either ND or IB viruses. A characteristic high mortality occurred in several trials. A small percentage of the embryos inoculated with IB virus were slightly dwarfed and curled but otherwise normal. It would appear that an acute generalized infection assaulted the embryos, resulting in death prior to the development of characteristic or diagnostic lesions. Loomis *et al.*[23] described extensive changes in chicken embryos infected with IB virus. In the latter embryos, perivascular granulocytes occurred in the liver with necrosis of entire lobes; the lungs were pneumonic and the kidneys showed interstitial nephritis.

Summary and Conclusions

The investigations pointed to the value of histopathological examination of infected embryos in the study of specific pathogens. Such studies may provide an insight into the pathogenesis of the agent, extend available diagnostic aids, and should be a valuable adjunct to established research methods.

By employing uniform methods and exsanguinating viable infected embryos, there was accomplished the identification of the principal organ or tissue attacked and the characteristic and pathognomonic lesion thus produced by four different avian pathogens (table 1).

Embryos infected with avian encephalomyelitis virus were grossly atrophied with an obvious reduction of skeletal musculature. The limbs were stiff and abnormally placed. A severe internal hydrocephalus occurred in the terminal stages of incubation. Specific histological lesions were observed in the nerve tissue of all infected embryos

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Fig. 12—Liver of 12-day-old embryo infected with avian PPLO. Many blood vessels are surrounded by heterophil aggregates. In the black and white photo, these cells appear similar to the reticular cells in figure 9. They are recognizable under the microscope by their small cytoplasmic eosinophilic granules, not rod-shaped as in postembryonic life. H & E stain; x 38.

Fig. 13—Lung of 18-day-old control embryo injected with diluent. Tertiary bronchi (A) and interstitial arterioles (B) are normal. H & E stain; x 145.

Fig. 14—Lung of 18-day-old embryo infected with avian PPLO. Tertiary bronchus is normal but interstitial arterioles are surrounded by heterophils. Lettering as in figure 13. H & E stain; x 145.

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TABLE 1—Principal Comparative Pathology of Avian Encephalomyelitis, Infectious Synovitis, Avian Infectious Hepatitis, and Pleuropneumonia-Like Organism Infections in the Developing Chicken Embryo

Microscopic lesions	Pathogen			
	AE*	Synovitis†	AIH**	PPLO
Hydrocephalus	++++	.....	.....	.....
Muscular dystrophy	++++	.....	.....	.....
Neuronal degeneration				
Spinal cord	+++	.....	.....	.....
Dorsal ganglia	+++	.....	.....	.....
Brain	++	.....	.....	.....
Ankylosis	+	.....	.....	.....
Reticular cell hyperplasia				
Liver	.....	++++	.....	.....
Gizzard	.....	++	.....	.....
Skeletal muscle	.....	+	.....	.....
Hepatic necrosis	.....	.....	++++	.....
Liver cord cell regeneration	.....	.....	++++	.....
Bile duct proliferation	.....	.....	++	.....
Bile stasis	.....	.....	++	.....
Hepatic heterophil infiltration	.....	.....	+	.....
Heterophilic perivasculitis				
Liver	.....	.....	.....	++++
Mesentery	.....	.....	.....	+++
Gizzard	.....	.....	.....	++
Skeletal muscles	.....	.....	.....	++
Loose connective tissue	.....	.....	.....	++
Lung (terminally)	.....	.....	.....	+

+ Estimated intensity of extent of lesions; * grossly inactive with muscular atrophy; † gross splenomegaly and hepatomegaly; ** livers green with creamy spots.

between postinoculation days 5 and 13. Neuronal degeneration was most conspicuous in the ventral horns of the spinal cord but extended to include all areas. Terminally, little or no functional nerve tissue remained in the spinal cord or brain. The inflammatory response in infected embryos was minimal and was limited to the brain and dorsal ganglia. Ankylosis of the femorotibial articulation occurred. The sympathetic nervous system, ependymal cells, and eyes failed to show changes.

Infectious synovitis virus caused hyperplasia of reticular cells around the blood vessels of the liver, gizzard, and skeletal muscles. The reticular cells were identified by cellular morphology, location, reaction to reticular fiber stains, and phagocytosis of trypan blue. No evidence of arthritis, tendovaginitis or bursitis was observed in infected embryos. Splenomegaly occurred.

Avian infectious hepatitis agent caused specific histological lesions consisting of focal necrosis with secondary diffuse or focal heterophilic infiltration and hepatic cell vacuolation. There was pronounced hepatic cord cell regeneration around the portal triads. Bile stasis and bile duct proliferation were extensive.

Avian pleuropneumonia-like organisms produced a heterophilic perivasculitis. Strains both with and without the ability to agglutinate chicken red blood cells produced the pathognomonic perivasculitis. Heterophilic perivasculitis was most prominent around the hepatic blood vessels but occurred also around the vessels of the gizzard, mesentery, skeletal muscles, and vessels permeating loose connective tissue throughout the body. Periarteritis in the lung developed only terminally in the incubation.

The embryonic response to infectious synovitis virus and avian infectious hepatitis agent was similar to that of the adult, while the response to avian encephalomyelitis and avian pleuropneumonia-like organism differed from that occurring in postembryonic life.

References

[1] Bardeen, C. R.: The Development of the Musculature of the Body Wall in the Pig, Including Its Histogenesis and Its Relations to the Myotomes and to the Skeletal and Nervous Apparatus. Bull. 9, Johns Hopkins Hosp., (1900): 367–400.

[2] Chute, H. L., and Cole, C. R.: Lesions in Chicken Embryos Produced by Pleuropneumonia-Like Organisms from Chronic Respiratory Disease of Chickens and Infectious Sinusitis of Turkeys. Am. J. Vet. Res., *15*, (Jan., 1954): 108–118.

[3] Danchakoff, V.: The Wandering Cells in the Loose Connective Tissue of Birds and Their Origin. Am. J. Anat., *20*, (1916): 483–492.

[4] Danchakoff, V.: Origin of the Blood Cells. Development of the Hematopoietic Organs and Regeneration of the Blood Cells from the Standpoint of the Monophyletic School. Am. J. Anat., *20*, (1916): 397–414.

[5] Danchakoff, V.: The Loose Connective Tissue as a Seat of Lymphogranulopoesis. Anat. Rec., *10*, (1916): 192–193.

[6] Danchakoff, V.: The Equivalence of Different Hematopoietic Anlages. I. Spleen. Am. J. Anat., *20*, (1916): 255–328.

XUM

[7] Dawson, J. R.: A Study of Chick-Embryo-Adapted Rabies Virus. Am. J. Path., *17*, (1941): 177–187.

[8] Delaplane, J. P., and Stuart, H. O.: The Propagation of a Virus in Embryonating Chicken Eggs Causing a Chronic Respiratory Disease in Chickens. Am. J. Vet. Res., *4*, (Oct., 1943): 325–333.

[9] Feibel, F., Helmboldt, C. F., Jungherr, E. L., and Carson, J. R.: Avian Encephalomyelitis—Prevalence, Pathogenicity, and Breed Susceptibility. Am. J. Vet. Res., *13*, (April, 1952): 260–266.

[10] Forsgren: Zeitschrift für Zellforschung und mikroskopische Anatomie. *6*, (1928): 649. [Cited by Gray, P.: The Microtomist's Formulary and Guide. Blakiston Co., Philadelphia, p. 423.]

[11] Glucksmann, A.: Über die Bedeutung von Zellvorgängen für die Formbildung epithelialer Organe. Ztschr. Anat., *93*, (1930): 35–93.

[12] Hamburger, V.: Die Entwicklung experimentell erzeugter nervenloser und schwach innervierter Extremitäten von Anuren. Arch. Entwickl.-Mech. Org., *114*, (1928): 273–363.

[13] Hamburger, V.: The Development and Innervation of Transplanted Limb Primordia of Chick Embryos. J. Exptl. Zoöl., *80*, (1939): 347–389.

[14] Hamburger, V., and Levi-Montalcini, R.: Proliferation, the Differentiation and Degeneration in the Spinal Ganglia of the Chick Embryo Under Normal and Experimental Conditions. J. Exptl. Zoöl., *111*, (1949): 457–502.

[15] Hamburger, V., and Waugh, M.: The Primary Development of the Skeleton in Nerveless and Poorly Innervated Limb Transplants of Chick Embryos. Physiol. Zoöl., *13*, (1940): 367–380.

[16] Hanan, E. B.: Absorption of Vital Dyes by the Fetal Membranes of the Chick. Am. J. Anat., *38*, (1927): 423–450.

[17] Herrmann, H.: Studies of Muscle Development. Ann. New York Acad. Sci., *55*, (1952): 99–108.

[18] Jungherr, E.: The Pathology of Experimental Sinusitis of Turkeys. Am. J. Vet. Res., *10*, (Oct., 1949): 372–382.

[19] Jungherr, E., and Minard, E. L.: The Present Status of Avian Encephalomyelitis. J.A.V.M.A., *100*, (Jan., 1942): 38–46.

[20] Jungherr, E., Sumner, F., Luginbuhl, R. E.: Pathology of Egg-Adapted Avian Encephalomyelitis. Science, *124*, (1956): 80–81.

[21] Koprowski, H., and Cox, H. R.: Studies on Rabies Infection in Developing Chicken Embryos. J. Bact., *54*, (1947): 74.

[22] Locy, W. A., and Larsell, O.: The Embryology of the Bird's Lung. Am. J. Anat., *19*, (1916): 447–504.

[23] Loomis, L. N., Cunningham, C. H., Gray, M. L., and Thorp, F.: Pathology of the Chicken Embryo Infected with Infectious Bronchitis Virus. Am. J. Vet Res., *11*, (July, 1950): 245–251.

[24] Love, R., and Roca-Garcia, M.: Pathology of Poliomyelitis in the Chick Embryo. Am. J. Path., *31*, (1955): 901–922.

[25] Love, R., and Roca-Garcia, M.: Pathogenesis of Poliomyelitis in the Chick Embryo. Arch. Path., *63*, (1957): 55–64.

[26] Sabin, F. R.: Direct Growth of Veins by Sprouting. Contrib. Embryol., *14*, (1922): 1–10.

[27] Scheidegger, S.: Experimental Viral Infections in the Embryo and Fetus. Preliminary Notes on Pathologic Findings with Viruses of Psittacosis, Ectromelia, and Rabies. Am. J. Path., *29*, (1953): 185–197.

[28] Sevoian, M., Snoeyenbos, G. H., Basch, H., and Reynolds, I.: Studies of Infectious Synovitis. Proc. Northeastern Conf. Workers Pullorum Dis. Control, 27th Ann. Meeting, mimeo. 1956.

[29] Sevoian, M., Winterfield, R. W., and Goldman, C. L.: Avian Infectious Hepatitis. I. Clinical and Pathological Manifestations. Avian Dis., *2*, (1958): 3–18.

[30] Sugiyama, S.: Origin of Thrombocytes and the Different Types of Blood Cells as Seen in the Living Chick Blastoderm. Contrib. Embryol., *18*, (1926): 121–148.

[31] Van Roekel, H., Gray, J. E., Shipkowitz, N. L., Clarke, M. K., and Luchini, R. M.: Chronic Respiratory Disease Complex in Chickens. Massachusetts Agric. Exper. Sta. Bull. 486, (1957): 68–69.

[32] Woodruff, A. M., and Goodpasture, E. W.: The Susceptibility of the Chorio-allantoic Membrane of the Chick Embryo to Infection with the Fowl Pox Virus. Am. J. Path., *7*, (1931: 209–222.

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Interference of Anti-Hog Cholera Hyperimmune Serum with the Immunity in Mice Vaccinated Against Swine Erysipelas with Adsorbed Bacterin

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SINCE 1951, vaccination of pigs against swine erysipelas has been practiced in Yugoslavia on a large scale exclusively with adsorbed bacterin. The degree of immunity was variable. In some areas the immunity was solid; in other areas poor, although the incidence of swine erysipelas was on about the same level. The immunity response was poor in areas where pigs were vaccinated simultaneously against swine erysipelas and hog cholera. Pigs in these areas were vaccinated against hog cholera with anti-hog cholera serum and virulent virus until 1955, and with serum and attenuated viruses after that time. When all factors concerning the variability in immunity response were considered, it was assumed that it was the hog cholera hyperimmune serum which interfered with the immunity against swine erysipelas.

To prove this hypothesis, experiments were carried out with mice. After preliminary experiments showed that anti-hog cholera serum neutralized the activity of adsorbed erysipelas bacterin, a number of samples of commercial anti-hog cholera serum were studied to determine whether they contained antibodies against swine erysipelas.

MATERIALS AND METHODS

The 11 lots of commercial anti-hog cholera serum used were specimens of lots already used or in preparation for use in the field. The serum had been produced from older pigs of different breeds. The pigs used for virus production originated from areas where hog cholera was absent but swine erysipelas was prevalent. Each lot of anti-hog cholera serum was a pooled sample of an average of 500 to 1,000 hogs.

The antibody content of serum samples was estimated in white mice (weight, 18 Gm.). The mice were inoculated subcutaneously with 0.04 to 1.28 ml. of serum. The protective power of the erysipelas component in the serum was not estimated in comparison to standard serum, but a 50 per cent mortality following challenge with virulent culture was calculated according to the method of Reed and Muench.[5] This method is, in our opinion, suitable for estimating a low antibody level. A protective unit of serum is the quantity which theoretically protects 50 per cent of the mice against a challenge dose of swine erysipelas culture. The relationship between the quantity of serum and the immunity units in 1 cc. of serum is shown (table 1).

TABLE 1—Method of Determining in Mice the Immunity Units Against Erysipelas in Anti-Hog Cholera Serum

Dose of serum to protect 1 mouse	No. of immunity units per ml.
0.04 ml.	25.0
0.08 ml.	12.5
0.16 ml.	6.25
0.32 ml.	3.1
0.64 ml.	1.56
1.28 ml.	0.78

Five mice were used to test each quantity of each lot of serum and, on the basis of accumulative deaths or survival of the mice, a 50 per cent mortality was calculated. As some mice given higher doses of serum developed an allergic reaction and died, they were replaced until 5 mice had been tested in each group.

To establish proof of the interference, a graphic method of logarithmic mesh was used according to Hazen.[1, 7]

In all experiments, only one specimen of a lot of adsorbed bacterin was used, and it was concentrated to one fifth of its original volume. In comparison with standard bacterin, this lot contained 83.3 protective units (p.u.). In calculating the effect of serum on the bacterin on the Hazen[1, 7] mesh, we plotted the values for the deaths of mice and set a line with two points (0.25 and 4.0 units of bacterin), as well as the values obtained when the mice were simultaneously inoculated with 1.0 unit of bacterin and the corresponding quantity of hog cholera serum.

In applying anti-hog cholera hyperimmune serum in mice, we tried to simulate an immunological relationship similar to that occurring in field

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vaccination of pigs against hog cholera (serum plus attenuated virus) and against swine erysipelas with adsorbed bacterin. It is assumed that, in the field, the dose is 5 ml. of adsorbed bacterin and an average of 10 ml. of anti-hog cholera serum per pig. On the basis of this dose, the following relationship between protective units of bacterin and immunity units (i.u.) in pigs and mice was calculated: Each pig vaccinated with 5 ml. of bacterin (83.3 p.u./ml.) and 10 ml. of anti-hog cholera serum (lot 60, 0.93 i.u./ml.) was given 416.6 p.u. of bacterin and 9.3 i.u. of swine erysipelas serum. A similar relationship in mice was simulated by injecting into them 0.022 ml. of serum for each protective unit (0.012 ml.) of bacterin. For each dose of serum and bacterin, a group of 30 mice was used.

To determine the titer of antibodies against swine erysipelas in anti-hog cholera serum, mice were challenged with swine erysipelas (strain S-38) 20 days after simultaneous inoculation of serum and adsorbed bacterin. Each mouse was inoculated subcutaneously with 0.2 ml. of a 24-hour nutrient broth diluted at $1:10^{-4}$. At this dilution, the strain killed mice in three to five days.

RESULTS

Determination of Titer of Antibodies in Anti-Hog Cholera Serums.—From 11 lots of examined serums, only one lot had a titer lower than 0.78 p.u., while in the remaining ten lots, the titer ranged from 0.93 to 4.65 units per milliliter (table 2).

The determined values of antibodies against swine erysipelas were obviously much lower in comparison with specific serums against swine erysipelas produced in horses or in pigs.[2]

In 54.5 per cent of examined serums (group 1 and 2) the titer of antibodies was low (0.78 to 1.20 i.u.), while only one lot had a titer of 4.65 i.u. per milliliter. When anti-hog cholera serums of group 4 were used for the immunization of pigs, it can be assumed that this serum interfered with the immunity against swine erysipelas produced with adsorbed bacterin.

Interference of Examined Lots of Hog Cholera Hyperimmune Serum with Immunity Produced by Adsorbed Bacterin.—As differences in antibody titers between lots in a group of serums were negligible, only one representative specimen from each group was examined: lot 60 (0.93 i.u.) from group 2; lot 8–12 (1.86 i.u.) from group 3; and lot 69, which showed the highest titer of antibodies (4.65 i.u.), from group 4.

Since the content of antibodies in these serums at least doubled in each ascending group, we expected to obtain proportional interference.

The mice were inoculated simultaneously with proportional quantities of adsorbed bacterin, which contained 83.5 p.u., and with one of these three anti-hog cholera serums. For each p.u. of bacterin (0.012 ml.), mice were injected with 0.022 ml. of serum from lot 66, 0.045 ml. of serum from lot 8–12 and 0.11 ml. of serum from lot 69.

TABLE 2—Antibody Titers Against Swine Erysipelas in Anti-Hog Cholera Serums

Serum group	No. of serum	No. of i.u.*/ml. of serum	Av. i.u. per group	Examined serum (%)
1	59	>0.78	>0.78	9.0
2	60	0.93		
	X	0.98		
	54	1.18	1.10	45.5
	IX	1.20		
	M	1.20		
3	8–12	1.86	1.94	18.2
	68	2.02		
4	70	3.12		
	77	3.12	3.63	27.3
	69	4.65		

* i.u. = immunity unit.

The results showed that, in order to achieve the same percentage of protection in a given percentage of mice inoculated with serum lot 60, mice given serum from lot 8–12 must be given 0.014 ml. of bacterin, and those given serum from lot 69 must be given 0.023 ml. of bacterin.

On the basis of these results, the active titer of adsorbed bacterin apparently diminished from 83.3 p.u. per milliliter, to 71.5 p.u., and to 43.4 p.u., respectively (table 3).

DISCUSSION

It is known from earlier studies that the immunizing capacity of adsorbed bacterin is diminished by a simultaneous use of anti-swine erysipelas hyperimmune serum. From our experiments, it is evident that anti-hog cholera serum can also interfere, depending on the quantity of immunity units against swine erysipelas which the anti-hog cholera serum contains.

The antibody titer against swine erysipelas in anti-hog cholera serum depends on several factors. For the production of anti-hog cholera serum, older pigs are used. They have been naturally immunized or vaccinated (sometimes twice) against swine erysipelas. Booster doses stimulate antibody production. The procedure of hog-

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cholera hyperimmunization may contribute to the increase of antibodies. In the U.S.A.,[6] it was found that approximately 5.9 per cent of the examined samples of virus blood contain erysipelas organisms. According to an observation in one of our commercial serum plants, there was gross evidence of swine erysipelas in about 5 per cent of slaughtered pigs used for the production of virus. Thus, since the blood of these pigs is not examined bacteriologically before inoculation in pigs used for the production of anti-hog cholera serum, the latter may be hyperimmunized with the culture of swine erysipelas, especially when the pigs used for anti-hog cholera serum production are reimmunized with virus, which is the routine practice in our serum plants.

Our experiments fully confirm this theory. Of 11 lots of anti-hog cholera serum, only one lot did not contain biologically demonstrable antibodies against swine erysipelas. All the remaining lots were found to contain sufficient antibodies to interfere with the immunity engendered by adsorbed bacterin. The results showed that serums which contained 2 i.u. per milliliter had no practical influence on the immunity response of adsorbed bacterin.

It is possible that this interference can be overcome with the adsorbed bacterins, which contain about 100 p.u. per milliliter. Meanwhile, anti-hog cholera serum, which contains 4.65 i.u. per milliliter, can completely inhibit development of immunity against swine erysipelas. Administration of 10 ml. of anti-hog cholera serum from lot 60 with adsorbed bacterin gave the pigs 357.5 p.u.; administration of the same quantity of serum from lot 69 gave the pigs only 297.0 p.u. The quantity of antibodies in 10 ml. of anti-hog cholera serum (46.4 i.u.) may have adverse effect on the immunity against swine erysipelas.

Observation in the field also supports this finding. Pigs, vaccinated simultaneously against swine erysipelas and hog cholera, when anti-hog cholera serum is used are practically protected against swine erysipelas during the negative phase, until the immunity is developed.

The interference can not be avoided even when the vaccination against swine erysipelas is performed 14 days after vaccination against hog cholera, because the hog-cholera serum is an homologous serum, which is excreted slowly.

The interference phenomenon should be avoided with new bacterins which have mineral oils[3,4] or saponin as adjuvants. In these bacterins, antigenic stimulation is prolonged beyond the time of serum elimination.

SUMMARY AND CONCLUSIONS

Immunity against swine erysipelas was less solid in areas of Yugoslavia where pigs were vaccinated simultaneously against erysipelas and cholera when anti-hog cholera serum was used.

Tests proved that ten of 11 commercial lots (91%) of anti-hog cholera examined contained antibodies against swine erysipelas. One lot (9%) had a titer of 4.65 immunity units and showed ability to lower the immunizing capacity of commercial adsorbed bacterin by 47 per cent.

Since anti-hog cholera serum, being homologous, is eliminated slowly, vaccination for erysipelas should either be postponed more than two weeks after its use, or a slowly adsorbed erysipelas vaccine should be used.

TABLE 3—Differences in Titer of Adsorbed Bacterin Caused by the Adverse Effect of Anti-Hog Chorela Serum

	Titer of bacterin administered with serum containing:					
	0.93 i.u./ml.		1.86 i.u./ml.		4.65 i.u./ml.	
No. p.u. in adsorbed bacterin/ml.	No. p.u.	Difference (%)	No. p.u.	Difference (%)	No. p.u.	Difference (%)
83.3	71.5	14.2	71.5	14.2	43.4	47.9

p.u. = protective unit; i.u. = immunity units.

References

[1] Eissner, G.: Messung der Wirksamkeit von Impfstoffen zur aktiven Schutzimpfung gegen Schweine Rotlauf. Monatsh. f. Prakt. Tierheilk., (Sept. 4, 1952): 401–413.

[2] Gelenczei and Rappay: Hyperimmunisierung von Pferden mit Rotlauf Bazillen-Stammen des A and B Typs. Acta vet. Acad. Sci. hung., (May, 1955): 1–9.

[3] Jungk, N. K., and Murdock, F. M.: An Emulsion-Type Erysipelas Bacterin. I. Duration of Immunity in Pigs Vaccinated at Weaning. Am. J. Vet. Res., *18*, (Jan., 1957): 121–125.

[4] Murdock, F. M., and Jungk, N. K.: An Emulsion-Type Erysipelas Bacterin. II. Duration of Immunity Following Vaccination of Newborn Pigs. Am. J. Vet. Res., *18*, (Jan., 1957): 126–132.

[5] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493–497.

[6] U.S. Department of Agriculture: Report, Agricultural Research Service, Washington, D.C., (1952): 209.

[7] Weber: Grundriss der Biol. Statist. Gustav Fischer. Jena, 1948.

Hematological Studies of Normal, Ascarid-Infected, and Hog Cholera-Vaccinated Swine

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WITH ADDITIONAL emphasis being placed by swine producers, livestock officials, veterinarians, and government agencies on the eradication of hog cholera, it has become imperative that research be accelerated in an attempt to answer many of the related perplexing problems confronting the swine industry. This project was undertaken because of the difficulty veterinarians sometimes encounter in vaccinating swine against hog cholera when using attenuated vaccines.

Field work has indicated that herds of swine, in which losses occurred following vaccination, frequently had severe ascarid infection and considerable damage had been done to the liver by the migrating larvae. An attempt has been made to investigate the effects of the lapinized hog cholera vaccines on young pigs, and to study the effects of ascarides (*Ascaris lumbricoides*) and their migrating larvae on the growing pig and their resulting influence, if any, on the immunizing potential of such infected pigs. Pathological findings will be reported in a later publication.

REVIEW OF LITERATURE

Hematological variations in normal swine have been reported (table 1). It appeared from this summary that the erythrocyte and leukocyte counts were lower in young than in old swine. The lymphocyte count increased with age at the expense of the neutrophils. The eosinophil count has been reported by Craft and Moe [13] to increase with age and by Gardiner *et al.*[16] to remain nearly the same. Basophils appeared to remain the same. Palmer [21] reported that there were no sex differences in differential or erythrocyte counts, but the leukocytes tended to be higher in male animals.

Eosinophilia has been reported to occur with ascarid infection.[3, 5, 9, 15, 22] Speirs and Driesbach [25] found that these cells concentrate around invading parasites, remain at the periphery of an inflamed area, and play only a minor role in phagocytosis of foreign material. It has been reported that the eosinophil plays some part in dealing with toxic materials, this material probably being derived from protein. Campbell [6] stated that the eosinophilia accompanying parasitic infection was due to the fact that the parasites had large amounts of keratin and that the keratin was responsible for an antigen-antibody reaction which caused the eosinophil increase. The antibody antigen reaction had large numbers of combining sites due to the wide distribution of the parasite throughout the body. Eosinophilia, then, was intimately associated with hypersensitivity. Campbell *et al.*[7] further stated that foreign proteins in themselves did not attract eosinophils, but that there must have been an allergic reaction beforehand, which stimulated the eosinophilia. The histamine released by antigen-antibody reactions was thought by Best and Taylor [2] to be the specific cause of eosinophil increases, and this was somewhat substantiated by the fact that histamine injections caused an eosinophilia similar to that caused by Ascaris-extract injections.

Most of the studies involving eosinophils with ascarids have been made using ascarid extracts on guinea pigs. This ascarid extract was an insoluble keratin-polysaccharide of high molecular weight. Campbell [6] found that intra-abdominal injections of 5 mg. of the extract per 100 Gm. of body weight elicited a rise to 2.5 per cent circulating blood-eosinophils and that 20 to 25 mg./100 Gm. of body weight caused a 25.0 per cent eosinophil count. In the latter case, the total leukocyte increase was 1,000 to 5,000 per cubic millimeter as a result of increases in the number of eosinophils and monocytes. Basophil numbers were increased in some animals. The eosinophil rise started in two to four days after the initial injection and reached its maximum in five to ten days. Antibodies against the extract were detected at the height of the reaction and for sometime afterward.

Vaughn [28] reported increases in blood eosinophils as early as six hours after injection of Ascaris extract into guinea pigs. Early pathological changes in the lungs were observed. The alveolar walls were thickened by swelling of the alveolar epithelium. This began in the larger vessels and bronchi and spread to involve large areas of the lungs and was followed by emigration of eosinophils from the blood vessels into the perivascular connective tissue. Eosinophils were later found in the pulp of the spleen.

Several investigators [24–28] have observed an in-

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TABLE 1—Summary of Reported Blood Studies of Swine of Various Ages

Author	Age	r.b.c. 10^6/cmm.	w.b.c. 10^3/cmm.	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)	Eosinophils (%)	Basophils (%)	Hematocrit (%)	Sedimentation (mm./hr.)
Albritton [1]	Adult	6.93	7.0–20.0	.....	.....	.....	.....	.....	30.0–53.0	3.0–8.0
	1–12 hours	5.51–5.91	.....	.....	.....	.....	.....	.....	39.0–40.0	.....
	1–10 days	2.62–5.26	.....	.....	.....	.....	.....	.....	18.0–36.0	.....
Coffin [10]	.....	5.0–9.0	8.6–20.0	40.0–60.0	1.0–10.0	30.0–50.0	1.0–10.0	0.0–4.0	32.0–47.0	1.0–14.0
Coles [12]	Adult	5.0–8.0	9.0–16.0	48.0–50.0	1.0–4.0	38.0–43.0	1.0–5.0	0.0	32.0–55.0	.....
Craft [13]	Birth	.....	.....	44.8	.....	54.9	0.1	.....	.....	.....
	30 days	.....	.....	62.3	.....	34.1	3.7	.....	.....	.....
	60 days	.....	.....	63.4	.....	27.3	9.2	.....	.....	.....
	90 days	.....	.....	61.0	.....	34.4	4.5	.....	.....	.....
	120 days	.....	.....	55.1	.....	41.1	3.6	.....	.....	.....
	180 days	.....	.....	64.9	.....	30.6	4.4	.....	.....	.....
Dinwiddie [14]	3–5 months	6.33	11.83	59.0	.....	35.0	3.0	0.5	.....	.....
Dukes [15]	.....	7.40	17.11	47.0	8.0	41.0	2.5	1.0	41.5	1.0–14.0
Gardiner [16]	1 day	.....	6.7–16.0	12.0–43.0	.....	54.0–87.0	.....	.....	33.0–44.0	.....
	8 days	.....	5.0–16.5	51.0–61.0	.....	34.0–48.0	.....	.....	27.0–35.0	.....
	15 days	.....	7.5–13.2	57.0–76.0	.....	22.0–44.0	.....	.....	28.0–47.0	.....
Giltner [18]	2.5–6 mo.	6.8–8.8	9.5–25.0	30.0–79.8	0.8–10.0	13.0–60.0	1.2–11.0	.....	.....	.....
Habel and	.....	5.7	14.7	38.0	4.0	53.0	4.0	.....	.....	.....
Biberstein [19]	.....	±0.7	±4.5	±13.0	±2.0	±11.0	±2.0	.....	.....	.....
Kernkamp [20]	Birth	5.5–7.0	9.0–16.0	.....	.....	.....	.....	.....	.....	.....
	7 days	3.0–4.5	9.0–16.0	.....	.....	.....	.....	.....	.....	.....
	14 days	5.0–6.5	9.0–16.0	69.0	.....	28.0	.....	.....	.....	.....
	3–6 weeks	5.5–7.0	9.0–16.0	.....	.....	.....	.....	.....	.....	.....
	7–12 weeks	6.0–7.0	14.0–20.0	.....	.....	.....	.....	.....	.....	.....
Palmer [21]	2–42 days	3.86	13.5	63.3	2.6	32.1	1.3	0.3	.....	.....
Runnells [22]	.....	6.70	15.9	52.0	3.0	39.0	5.0	1.2	.....	.....
Shu [23]	.....	6.14–8.12	17.3–26.2	34.0–58.0	0.0–2.0	25.0–55.0	4.0–22.0	0.0–2.5	.....	.....
Swenson	36 hours	5.10	7.36	.....	.....	.....	.....	.....	33.9	.....
et al.[27]		±1.13	±1.81	.....	.....	.....	.....	.....	±4.9	.....

crease in eosinophils in guinea pigs following the injection of an ascaris extract. They found that upon a second injection of the extract into a sensitized guinea pig, there was a tremendous increase in eosinophils at the site of injection. The degree of concentration, then, depended upon the amount of prior sensitization.

If the keratin portion of the ascarid is responsible for the eosinophilia, then after death of the parasite a greater eosinophilia may be expected, as the keratin portion of the parasite is more available after death. This was confirmed by Campbell.[6] In hookworm infection, eosinophilia regularly occurred only after death of the parasites.[3] If a large number of the parasites entered the host and died before reaching the intestine, a greater eosinophilia may have resulted. If all larvae successfully reached their adult habitat, no eosinophilia occurred.

Reports on the chemical blood changes accompanying ascariasis have been published.[4] Cole [11] found leukopenia to be a regular phenomenon following vaccination with antiserum and virulent virus (simultaneous method), and in many cases it was then followed by a leukocytosis which resulted in a higher leukocyte count than was present before vaccination. His experiment (on 55–90 lb. pigs, using 35 cc. of serum and 2 cc. of virus) indicated a decrease of from 8,000 to 20,000 circulating leukocytes in three to five days following vaccination. These counts returned to normal by the sixth to seventh day and exceeded the original counts in some cases by the eighth day. The leukopenia, following the simultaneous vaccination, became less severe as the dose of serum was increased, and more severe as the dosage of serum was decreased. Serum given alone did not cause a reduction in leukocytes.

MATERIALS AND METHODS

Seven pregnant sows of mixed breeding were obtained, and a total of 60 pigs were farrowed, of which 40 were saved for this project. Each pig was numbered and ear-notched accordingly.

The pigs were allotted to four groups. Groups 1 and 2 were arranged so that principal and control animals were litter mates and suckled the same mother. Group 1 contained 2 controls and 4 principal animals, and group 2 was composed of 4 controls and 10 principals.

Groups 3 and 4 were divided into control and principal animals by litters, the principal animals having no contact with the controls. Group 3 contained 4 controls and 5 principal animals, and group 4 contained 7 controls and 9 principals.

Sows and pigs were kept in houses with concrete floors and walls, the pens being completely indoors. The floors were cleaned twice each day and hosed with water once each day. A 16 per cent protein, complete commercial ration was fed prior to farrowing and for the duration of the experiment to both sows and pigs.

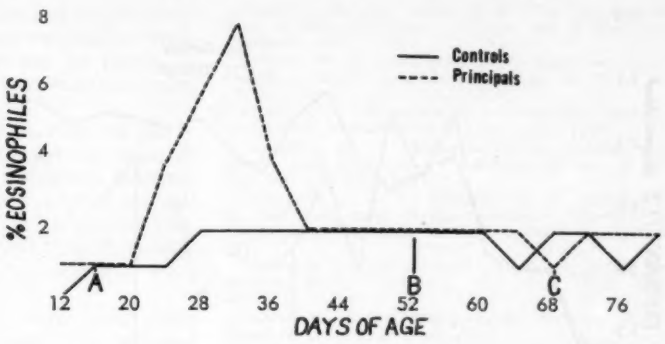
Blood samples were collected aseptically from the anterior vena cava according to the procedure described by Carle and Dewhirst.[8] The citrated blood was analyzed within the next few hours by methods described by Coffin [10] for total erythrocyte and leukocyte numbers, hematocrit, sedimentation rate, and differential leukocyte counts. Blood smears were made at the time samples were collected, and were stained with Giemsa's stain within 48 hours. Only cells showing nuclear fragments separated by extremely thin filaments were considered segmented neutrophils.

The first samples were collected seven to 14 days after birth, and the second was taken four days

TABLE 2—Summary of Blood Studies of 12 Control Pigs

Age (days)	r.b.c. 10^6/cmm.	w.b.c. 10^3/cmm.	Sedimentation mm./hr.	Hematocrit (%)	Polymorphonuclear (%)	Stab (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)
10	4.71	7.53	2.64	20.4	17.0	4.0	78.0	1.0	....	....
14	4.78	11.19	1.27	21.8	19.0	8.0	73.0	1.0	1.0	....
18	5.27	10.32	1.83	23.4	18.0	6.0	72.0	2.0	1.0	....
22	5.39	10.87	1.76	24.5	17.0	18.0	65.0	....	....	....
26	5.48	12.65	2.00	25.2	13.0	16.0	69.0	2.0	....	....
30	5.93	12.28	1.77	25.5	17.0	14.0	66.0	1.0	2.0	....
34	5.66	10.88	1.54	27.1	13.0	14.0	71.0	1.0	1.0	....
38	6.24	14.90	1.19	27.4	12.0	20.0	65.0	1.0	2.0	....
42	5.67	13.00	1.32	26.7	11.0	18.0	69.0	1.0	1.0	....
46	6.38	14.17	0.28	26.9	9.0	22.0	67.0	1.0	1.0	....
50	6.32	15.30	1.27	27.8	8.0	24.0	66.0	1.0	1.0	....
54	6.60	14.50	1.33	29.7	10.0	21.0	65.0	2.0	3.0	....
58*	6.16	14.64	1.96	29.0	9.0	23.0	65.0	2.0	1.0	....
62	6.65	13.82	2.09	30.1	13.0	19.0	64.0	1.0	3.0	....
66	6.42	15.67	2.30	30.9	12.0	22.0	62.0	1.0	1.0	....
70	6.25	15.77	1.76	31.5	10.0	22.0	63.0	3.0	2.0	....
74†	6.51	16.46	2.03	29.9	8.0	17.0	72.0	2.0	1.0	....
78	6.37	14.96	1.45	29.8	10.0	21.0	67.0	1.0	1.0	....
82	6.51	14.30	1.82	30.0	11.0	20.0	69.0	....	....	....
86	7.29	16.08	2.19	32.0	11.0	20.0	66.0	1.0	2.0	....
90	7.15	17.26	2.28	33.0	2.0	18.0	77.0	2.0	1.0	....
94	7.63	16.82	1.90	32.5	9.0	34.0	55.0	1.0	1.0	....

* Vaccination with modified hog cholera vaccine occurred at this time. † Challenge with live hog cholera virus occurred at this time.



Graph 1—Averages of eosinophil counts during the course of the experiment. (A) ascarid inoculation of principal animals; (B) vaccination of all animals with lapinized hog cholera vaccine without serum; (C) challenge of all animals with 4 cc. of virulent hog cholera virus.

later, at the time of ascarid egg inoculation. These samples were used for a base line. Thereafter, blood samples were obtained at intervals of four days from both control and principal animals until their death or termination of the project.

Female ascarides were obtained locally from slaughtered swine. The uteri from mature worms were removed and the eggs expelled into sterile Petri dishes, where they were mixed with a small quantity of tap water (approximately 100 cc.). One drop of commerical merthiolate per dish was added to retard mold growth. This material was transferred to cotton-stoppered Erlenmeyer flasks (250 cc.) containing glass beads. These flasks were then incubated at room temperature with a minimum of light. They were agitated once a week and the progress of the embryos was observed. After the eggs had become embryonated, a period of six weeks or more allowed to elapse before their use.

A human catheter was passed into the stomach of each principal pig and a culture, previously computed by direct microscopic count of a representative sample, was transferred into the stomach. Control animals were not inoculated. Principal animals from groups 1 and 2 were inoculated with 165,000 live embryonated ova. Groups 3 and 4 were given 125,000 live embryonated ova. These inoculations were made at approximately the same time as the second blood collections (12 to 18 days of age).

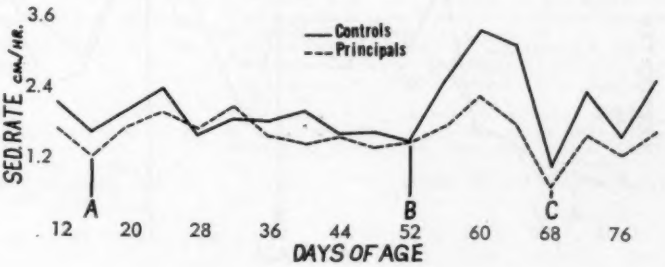
At approximately 8 weeks of age, all remaining principal and control pigs were vaccinated subcutaneously with 2 cc. of rabbit-modified, live-virus hog cholera vaccine. No hyperimmune serum was administered. Sixteen to 21 days from this date, a challenge dose of 4 cc. of commerical virulent hog cholera virus was given subcutaneously (2 cc. was lethal to susceptible pigs within 5 days).

RESULTS

Normal Pigs.—Results of blood studies of 12 normal, healthy pigs which served as control animals for this experiment are summarized (table 2). Control animals which were not considered clinically "normal" are not included.

There was a gradual rise in erythrocyte counts in pigs 10 days to 7 weeks old, after which fluctuations occurred with slight elevations in total numbers. Total leukocyte counts also rose during this periods; after the pigs were approximately 50 days old, a fluctuating level was attained. Differential counts revealed an increase in neutrophils at the expense of lymphocytes. Monocytes and eosinophils remained fairly constant. Basophils were few. Sedimentation rates varied from 0.28 cmm. per hour to 2.6 cmm. per hour, with no apparent pattern. Hematocrit readings corresponded closely with total erythrocyte counts.

These findings varied from those previously reported in studies of the blood of the



Graph 2—Averages of sedimentation rates during the course of the experiment. (A) ascarid inoculation of principal animals; (B) vaccination of all animals with lapinized hog cholera vaccine without serum; (C) challenge of all animals with 4 cc. of virulent hog cholera virus.

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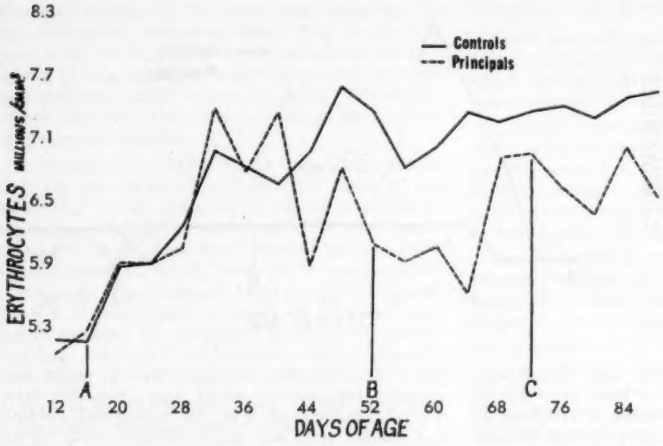


Graph 3—Averages of erythrocyte counts during the course of the experiment. (A) ascarid inoculation of principal animals; (B) vaccination of all animals with lapinized hog cholera vaccine without serum; (C) challenge of all animals with 4 cc. of virulent hog cholera virus.

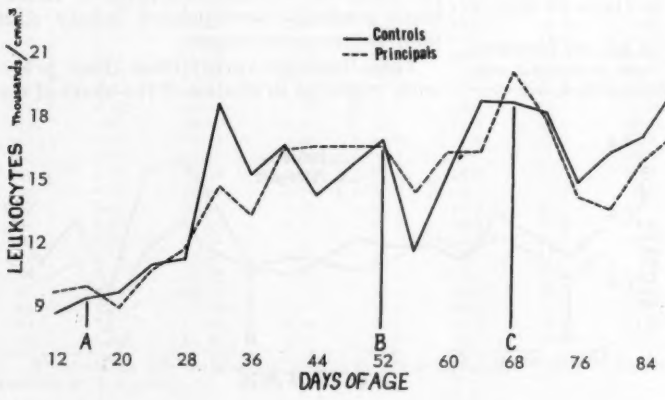
normal pig, chiefly because of an increase in neutrophils with a corresponding decrease in lymphocytes. Hematocrit readings were generally lower than previously reported, but total erythrocyte counts were essentially the same.

Ascarid-Infected Pigs.—Eosinophil counts increased in ascarid-infected animals by the fourth day postinfection and continued to increase until 12 to 16 days. Then the counts decreased to preinfective levels by 20 to 28 days (graph 1).

Sedimentation rates increased only slightly in the principal pigs following ascarid infection. Hog cholera vaccination and challenge of principal and control animals increased the sedimentation rates. The increase following immunization was greater than that following challenge (graph 2).

Routine blood studies in these experiments indicated that a mild anemia accompanied the ascarid infection (graph 3). Hematocrit determinations corresponded with these findings.

Pigs Vaccinated with Lapinized Vaccines.—Blood studies indicated that following vaccination with modified vaccines, and after challenge with virus, a transitory leukopenia resulted (graph 4). This was not a severe leukopenia and was similar to that which was found in the work of commercial



Graph 4—Averages of leukocyte counts during the course of the experiment. (A) ascarid inoculation of principal animals; (B) vaccination of all animals with lapinized hog cholera vaccine without serum; (C) challenge of all animals with 4 cc. of virulent hog cholera virus

companies,[17] although detailed information on blood studies following vaccination with lapinized vaccines could not be found or was not made available from reluctant commercial firms.

The mild leukopenia found in this study existed for a longer period in ascarid-infected animals than in control animals. When a challenge dose of live virus was administered to these animals, a similar syndrome was observed, except that the leukopenia was of a longer duration and even less severe.

Ascarid-infected pigs recovered more slowly from the leukopenia that followed immunization and challenge than did the control animals (graph 4).

SUMMARY

The hematology of normal young pigs has been reported and compared with previously published results. Total leukocyte and erythrocyte counts increased from birth to approximately 7 weeks of age. The ratio of neutrophils increased at the expense of lymphocytes, contrary to previous reports.

Pigs experimentally infected with ascarid ova developed a mild anemia that was noticeable 32 days after infection. Circulating eosinophils increased four days postinfection, reached a peak by the sixteenth day, and returned to normal levels within 28 days.

Pigs immunized with rabbit-modified hog cholera vaccine used without serums developed a mild leukopenia following both vaccination and challenge. The leukopenia following vaccination lasted for approximately eight days, whereas that following challenge existed for at least 16 days. A leukocytosis developed following the vaccination leukopenia in these pigs. The leukopenia existed for a longer time in ascarid-infected animals, and the count was slower in returning to normal than it was in the control pigs.

These observations may be one of the explanations for postvaccination difficulties encountered by veterinarians in the field with some lots of pigs.

References

[1] Albritton, E. C.: Standard Values in Blood. W. B. Saunders Co., Philadelphia, 1952.

[2] Ashford, B. K., Payne, G. C., and Payne, F. K.: Acute Uncinariasis from Massive Infestation and Its Complications. J. Am. M. A., *101*, (1933): 843.

[3] Best, C. H., and Taylor, N. S.: The Physiological Basis of Medical Practice. Williams and Wilkins Co., Baltimore, 1955.

[4] Biester, H. E., and Eveleth, D. F.: Blood and Tissue Studies in Experimental Ascariasis. Am. J. Hyg., *25*, (1937): 135.

[5] Boyd, W. C.: Fundamentals of Immunology. 2nd ed. Interscience Publishers, New York, N.Y., 1947.

[6] Campbell, D. H.: Experimental Eosinophilia with Keratin from *Ascaris Suum* and Other Sources. J. Infect. Dis., *71*, (1942): 270.

[7] Campbell, A. C. P., Drennan, A. M., and Rettie, T.: The Relationship of the Eosinophilic Leucocyte to Allergy and Anaphylaxis. J. Path. & Bact., *40*, (1935): 537.

[8] Carle, B. N., and Dewhirst, W. H.: A Method for Bleeding Swine. J.A.V.M.A., *101*, (Dec., 1942): 495–496.

[9] Carpenter, P. L.: Immunology and Serology. W. B. Saunders Co., Philadelphia, 1956.

[10] Coffin, D. L.: Manual of Veterinary Clinical Pathology. Comstock Publishing Associates, Ithaca, N.Y., 1953.

[11] Cole, C. G.: Leucocyte Counts on the Blood of Normal, Cholera Infected, and Recently Immunized Pigs. J.A.V.M.A., *81*, (Sept., 1932): 392–400.

[12] Coles, E. H.; Kansas State College: Unpublished data, 1957.

[13] Craft, W. A., and Moe, L. H.: Statistical Observations Involving Weight, Hemoglobin, and the Proportion of White Blood Cells in Pigs. J.A.V.M.A., *81*, (Sept., 1932): 405–407.

[14] Dinwiddie, R. R.: Studies on the Hematology of Normal and Cholera Infected Hogs. Univ. Arkansas Agric. Exper. Sta. Bull. 120, 1914.

[15] Dukes, H. H.: The Physiology of Domestic Animals. 7th ed. Comstock Publishing Associates, Ithaca, N.Y., 1955.

[16] Gardiner, M. R., Suppel, W. L., and McCormick, W. C.: The Blood Picture in Newborn Pigs. Am. J. Vet. Res., *14*, (Jan., 1953): 68–71.

[17] Gellen, V. W.: The Newly Vaccinated Pig. Corn States Needle, *31*, (1956): 3.

[18] Giltner, W.: The Histology and Physiology of Normal Pigs' Blood. J. Comp. Path. & Therap., *20*, (1907): 18.

[19] Habel, R. E., and Biberstein, E. L.: Fundamentals of the Histology of Domestic Animals. Comstock Publishing Associates, Ithaca, N.Y., 1952.

[20] Kernkamp, H. C. H.: The Blood Picture of Pigs Kept Under Conditions Favorable to the Production and to the Prevention of So-called "Anemia of Suckling Pigs." Univ. Minnesota Agric. Exper. Sta. Tech. Bull 86, 1932.

[21] Palmer, C. C.: Morphology of Normal Pigs' Blood. J. Agric. Res., *9*, (1917): 131.

[22] Runnells, R. A.: Animal Pathology. 5th ed. Iowa State College Press, Ames, 1954.

[23] Shu, S.: Studies on the Cellular Changes in Pigs' Blood During the Development of Hog Cholera. Ann. Rep. New York State Vet. Coll., 1930.

[24] Speirs, R. S.: Physiological Approaches to an Understanding of the Function of Eosinophils and Basophils. Ann. N.Y. Acad. Sci., *59*, (1955): 706.

[25] Speirs, R. S., and Dreisback, M. E.: Quantitative Studies of the Cellular Responses to Antigen Injections in Normal Mice. Blood, *11*, (1956): 44.

[26] Spcirs, R. S., Wench, U., and Dreisback, M. E.: Quantitative Studies of the Cellular Responses to Antigen Injections in Adrenalectomized Mice. Blood, *11*, (1956): 56.

[27] Swenson, M. J., Goetsch, D. D., and Underbjerg, G. K. L.: The Effect of the Sow's Ration on the Hematology of the Newborn Pig. Proc. Book, AVMA, (1955): 159.

[28] Vaugn, J.: The Function of the Eosinophilic Leucocyte. Blood, *8*, (1953): 1.

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The Use of the Plaque Technique for the Study of Porcine Enteroviruses

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THE OBSERVATION by Koch that bacteria would produce "colonies" on solid mediums, and thus could be isolated and studied in pure culture, paved the way for a rapid advance in bacteriology. Similarly, the observation by Dulbecco [5] that certain animal viruses are capable of producing plaques on monolayer cultures of mammalian cells grown *in vitro* has made available to the virologist a technique comparable to the one which has been used so successfully by the bacteriologist.

The purpose of this paper is to describe how the plaque technique may be used for the study of the porcine enteroviruses.

REVIEW OF LITERATURE

Although some animal viruses produce plaques on the chorioallantoic membranes of chicken embryos,[3] the use of this technique is definitely limited as compared with the method developed by Dulbecco,[5] wherein tissue culture methods are utilized. Since the advent of the Dulbecco plaque technique, it has been used successfully to obtain information on many viruses, including the human enteroviruses such as poliomyelitis [6] enteric cytopathogenic human orphan (ECHO),[10, 13] and Coxsackie.[10]

The plaque technique has been found to be admirably suited for conducting the following studies on viruses: adsorption onto cells,[10] isolation, [12, 13] obtaining pure lines,[6] differentiation and identification by type of plaque produced,[4, 11, 12] enumeration,[1, 6] neutralization,[8] mutation,[15] and growth curves.[1, 7]

No reference could be found where the plaque technique has been used for the study of the intestinal viruses of swine, although tissue culture methods have been used for the isolation of such viruses. Moscovici *et al.*[14] reported, from Italy, the isolation of a virus from an epizootic of enteritis in newborn pigs. This agent produced a cytopathogenic effect on porcine and monkey kidney cells. Beran *et al.*,[2] using tissue culture methods, isolated a number of cytopathogenic agents from the feces of pigs. The majority of these agents belonged to a single homotypic group. Hancock *et al.*[9] isolated 30 unidentified viruses from eight swine herds by the use of porcine kidney tissue cultures. Preliminary studies indicated that four of these were of different types.

MATERIALS AND METHODS

Preparation of Tissue Culture Bottles.—The tissue culture methods used for growing the porcine kidney cells were essentially those described in a previous paper.[9] The trypsinized, washed, porcine kidney cells were suspended in the culture medium in a dilution of 1:200, and approximately 15 ml. was inoculated into 4-oz. prescription bottles. The medium was changed after about three days. In most cases, the bottles contained a confluent sheet of cells after six or seven days, and were then suitable for use with the plaque technique.

The growth medium consisted of 0.5 per cent lactalbumin hydrolysate, 5.0 per cent bovine serum, and 94.5 per cent Hanks' balanced salt solution (BSS). Antibiotics were added to give a final concentration of 200 units of penicillin, 200 μg. of dihydrostreptomycin, and 50 units of nystatin (Mycostatin)* per milliliter of medium. Sodium bicarbonate (4.4%) was added to give the desired pH (6.9 to 7.1 for initiating growth and 7.6 to 7.8 for maintaining growth).

Agar Overlay Medium.—To 100 ml. of BSS was added 0.5 Gm. lactalbumin hydrolysate and 1.0 Gm. of Noble agar (Difco). This was autoclaved at 7 lb. of pressure for ten minutes and then placed in a water bath and cooled to 42 C. The following reagents were then added: 3 ml. of bovine serum, 1 ml. of a 1:1,000 dilution of neutral red, 6 ml. of a 4.4 per cent solution of sodium bicarbonate, and antibiotics to give the concentration previously described. By this procedure, the pH of the medium was usually 7.5.

Plaque Technique.—Our technique was similar to the one used by Hsiung and Melnick.[10] Only those bottles showing a confluent sheet of healthy cells were used. Prior to adding the virus, the fluid in the bottle was removed. The inoculum for each bottle usually consisted of 0.2 ml. of the virus material, and an attempt was made to disperse the inoculum over the cellular monolayer by tilting the bottles back and forth several times. The inoculated bottles were incubated at 37 C. for one

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* Mycostatin is produced by E. R. Squibb & Sons, New York, N.Y.

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Fig. 1—Plaques resulting from the inoculation of a fecal suspension obtained from a pig.

hour, during which time it was expected that most of the viral particles would be adsorbed onto the cells.

About 12 to 15 ml. of the agar overlay medium (at 42 C.) was then added to each bottle, which was placed on its side so that the agar solidified over the cellular monolayer. In some cases, just prior to adding the agar, the bottles were rinsed several times with BSS to remove any unadsorbed virus. After the agar solidified, the bottles were inverted and incubated at 37 C.

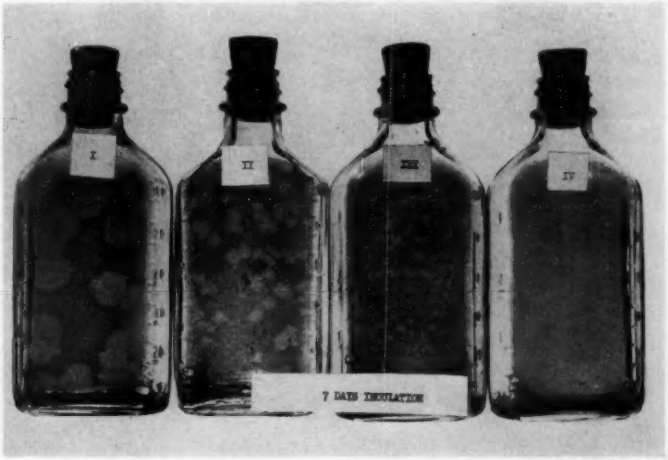
Preparation of Fecal Samples for Virus Isolation.—A small quantity of feces was collected from individual pigs by the use of a rectal swab, which was then immediately placed in a tube containing 3 ml. of BSS with the following concentration of antibiotics per milliliter: 2,000 units of penicillin, 1,000 μg. of dihydrostreptomycin, and 500 units of nystatin. The fecal suspension was then centrifuged in an angle-head centrifuge at a speed of 3,500 r.p.m. for 40 minutes.

Viruses.—Some of the porcine enteroviruses used in this study have been reported in a previous publication.[9] The pseudorabies virus was originally obtained from the American Type Culture Collection,* and the tenth passage in porcine kidney tissue culture was used. The infectious canine hepatitis virus,† as used in the study, represented the eighth passage in porcine kidney tissue culture. The transmissible gastroenteritis (TGE) virus was a fecal suspension from known infected baby pigs.

EXPERIMENTAL PROCEDURE AND RESULTS

Isolation of Viruses from Fecal Suspensions.—Fecal suspensions were inoculated into culture bottles in 0.2 ml. amounts, and the plaque technique was then followed. Observations were made daily for the appearance of plaques. An example of the direct isolation, from porcine feces, of a virus in the form of isolated plaques is

* American Type Culture Collection, Washington, D.C.
† Obtained through the courtesy of Dr. Victor Cabasso, Lederle Laboratories, Pearl River, N.Y.



—Ohio State University

Fig. 2—Bottles I, II, and III show three different types of plaques resulting from the growth of three different porcine enteroviruses after seven days of incubation. Bottle IV is a control (no virus).

illustrated (fig. 1). This photograph was made after an incubation period of five days, although the plaques first became visible after 48 hours.

Some porcine enteroviruses isolated by this technique produce grossly visible plaques in 24 hours, while others require about seven days. Isolation of a virus from an individual plaque can be made by transferring a small amount of the plaque material to tissue culture tubes with a capillary pipette.

Isolation of Pure Lines of Viruses.—Since a single plaque, in most cases, arises from a single virus particle, it is possible by this technique to isolate and propagate a pure line of virus.

The following procedure was used for obtaining pure lines of porcine enteroviruses. The virus sample, properly diluted, was inoculated into tissue culture bottles. After incubation at 37 C. for one hour, each bottle was rinsed three times with 10 ml. of BSS to remove any unadsorbed virus. Agar overlay medium was then added and the bottles were incubated at 37 C. Bottles which showed only one or two plaques were then selected for further work. An agar core from the periphery of the plaque was obtained by means of a capillary pipette and transferred to a tissue culture tube. Care was then taken not to touch the water of condensation with the pipette. When the inoculated tissue culture showed a marked cytopathogenic effect, the fluid, which was considered to represent a pure line of the virus, was harvested.

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Fig. 3—Plaques resulting from the inoculation of 10^{-6} (four plaques) and 10^{-5} (approximately 48 plaques) dilutions of a virus.

Differentiation of Viruses by the Type of Plaque Produced.—Some of the viruses

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Fig. 4—Serum-neutralization test. Serum A neutralized the virus so that no plaques developed.

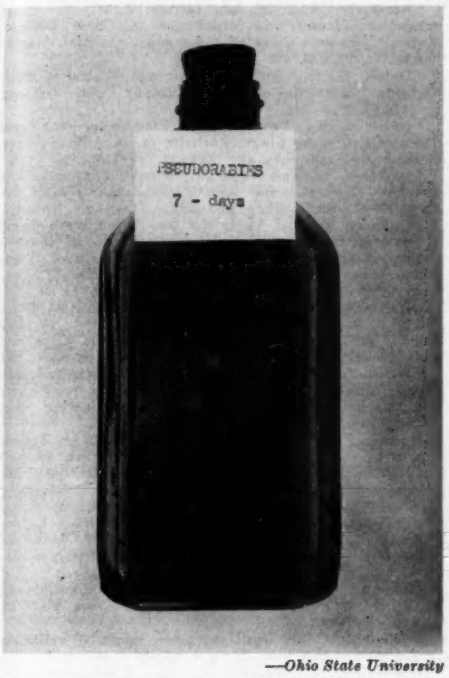

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Fig. 5—Plaques of pseudorabies after seven days of incubation.

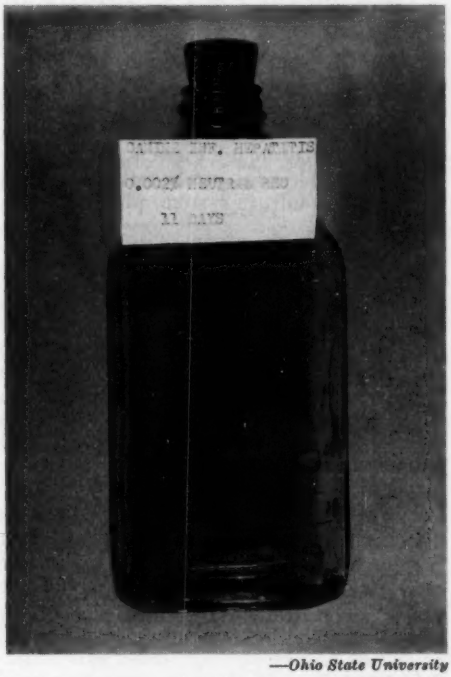

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Fig. 6—Plaques of infectious canine hepatitis after 11 days of incubation.

isolated from the feces of swine produced different types of plaques (fig. 2). A few of the more obvious differences that have been observed are: (1) incubation time necessary for the first appearance of the plaque; (2) the diameter of the plaque, or how rapidly it increased in size; and (3) the type of border shown by the plaque.

Enumeration of Viruses.— By inoculating a tissue culture bottle with 0.2 ml. of a known dilution of the virus or fecal sample, and by counting the number of plaques arising in the bottle, the number of "plaque-forming units" (PFU) in the original material can be determined. However, in order to get discrete and, thus, countable plaques, a suitable dilution of the virus sample must be used. The number of plaques that was obtained with 10^{-4} and 10^{-5} dilutions of a virus sample is illustrated (fig. 3).

Neutralization Tests.—The results of a serum-neutralization test utilizing the plaque technique is shown (fig. 4). In this case, 1.0 ml. of a suitable virus dilution (previously determined to have contained about 50 PFU per 0.1 ml.) was placed in each of three tubes. To these three tubes were added: tube 1, 1.0 ml. of BSS; tube 2, 1.0 ml. of a 1:32 dilution of serum A; tube 3, 1.0 ml. of a 1:32 dilution of serum B. These tubes were incubated at room temperature for one hour, and then 0.2 ml. of the contents of each tube was inoculated into a tissue culture bottle and assayed for the presence of virus. It is obvious (fig. 4) that serum A contained a neutralizing substance (in this case, antibody) for the virus.

Known Viruses.—Pseudorabies virus and infectious canine hepatitis virus also produced plaques on porcine kidney cells (fig. 5, 6), while TGE virus failed to do so.

DISCUSSION

In many respects, the development of plaques by viruses is analogous to the development of colonies by bacteria. The Dulbecco plaque technique provides a new and valuable tool for the virologist, and will undoubtedly contribute greatly to the advances in virology.

This report indicates that the plaque technique can be used successfully for the study of the porcine enteroviruses. However, one limitation, at present, is the fact that not all viruses produce plaques. For example, no plaques were produced with the TGE virus, although there is a possibility that if suitable susceptible cells were used in the tissue culture system, plaques might then develop.

SUMMARY AND CONCLUSIONS

1) The Dulbecco plaque technique, utilizing porcine kidney cells, was used successfully for the study of the porcine enteroviruses in: *(a)* primary isolation from feces, *(b)* obtaining pure lines from single plaques, *(c)* differentiating strains on the basis of plaque characteristics, *(d)* enumerating, and *(e)* conducting either virus- or serum-neutralization tests.

2) The viruses of pseudorabies and canine infectious hepatitis produced plaques on porcine kidney cells.

3) The virus of transmissible gastroenteritis of swine did not produce plaques on porcine kidney cells.

References

[1] Bachrach, H. L., Callis, J. J., Hess, W. R., and Patty, R. E.: A Plaque Assay for Foot-and-Mouth Disease Virus and Kinetics of Virus Reproduction. Virology, *4*, (1957): 224–236.

[2] Beran, G. W., Werder, A. A., and Wenner, H. A.: Enteroviruses of Swine. I. Their Recognition, Identification, and Distribution in a Herd of Swine. Am. J. Vet. Res., *19*, (July, 1958): 545–553.

[3] Beveridge, W. I. B., and Burnet, F. M.: The Cultivation of Viruses and Rickettsiae in Chick Embryo. Medical Research Council (London), Special Report Series No. 256, 1946.

[4] Dubes, G. R.: Differences Among Strains of Polio Virus in Plaque Size on Monkey Kidney Cells. Virology, *2*, (1956): 284–288.

[5] Dulbecco, R.: Production of Plaques in Monolayer Tissue Cultures by Single Particles of an Animal Virus. Proc. Natl. Acad. Sci., *38*, (1952): 747–752.

[6] Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., *99*, (1954): 167–182.

[7] Dulbecco, R., and Vogt, M.: One-Step Growth Curve of Western Equine Encephalomyelitis Virus on Chicken Embryo Cells Grown *in Vitro* and Analysis of Virus Yields from Single Cells. J. Exptl. Med., *99*, (1954): 183–199.

[8] Dulbecco, R., Vogt, M., and Strickland, A. G. R.: A Study of the Basic Aspects of Neutralization of Two Animal Viruses, Western Equine Encephalitis Virus and Poliomyelitis Virus. Virology, *2*, (1956): 162–205.

[9] Hancock, B. B., Bohl, E. H., and Birkeland, J. M.: Swine Kidney Cell Cultures: Susceptibility to Viruses and Use in Isolation of Enteric Viruses of Swine. Am. J. Vet. Res., *20*, (Jan., 1959): 127–132.

[10] Hsiung, G. D., and Melnick, J. L.: Plaque Formation with Poliomyelitis, Coxsackie, and Orphan (ECHO) Viruses in Bottle Cultures of Monkey Epithelial Cells. Virology, *1*, (1955): 533–535.

[11] Hsiung, G. D., and Melnick, J. L.: Morphological Characteristics of Plaques Produced on Monkey Kidney Monolayer Cultures by Enteric Viruses (Poliomyelitis, Coxsackie, and ECHO Groups). J. Immunol., *78*, (1957): 128–136.

[12] Hsiung, G. D., and Melnick, J. L.: Orphan Viruses of Man and Animals. Ann. New York Acad. Sci., *70*, (1958): 342–361.

[13] Melnick, J. L.: ECHO Viruses. Special Publication of the New York Acad. Sci., *5*, (1957): 367–392.

[14] Moscovici, C., Ginevri, A., and Massaracchio, V.: Isolamenta so Coltura di Tessuti di un Virus da Enteriti Suina. Zooprofilassi, *1*, 1956.

[15] Vogt, M., Dulbecco, R., and Wenner, H. A.: Mutants of Poliomyelitis Viruses with Reduced Efficiency of Plating in Acid Medium and Reduced Neuropathogenicity. Virology, *4*, (1957): 141–155.

[16] Younger, J. S.: Virus Adsorption and Plaque Formation in Monolayer Cultures of Trypsin-Dispersed Monkey Kidney. J. Immunol., *76*, (1956): 288–292.

The Changing Morphology in the Uterus of the Growing Pig

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THE PURPOSE of this work was to attempt to tabulate the influence of age on the uterine morphology in swine. There are a number of papers dealing with the alterations of uterine morphology and histochemistry [1, 4, 6] in the different phases of the sexual cycle and pregnancy but, to our knowledge, none report an attempt to study the influence of age on uterine morphology.

MATERIALS AND METHODS

Specimens were collected, during 1954 and 1955, from 38 females representing 16 different age groups, from the newborn pig to sows 33 months old (table 1).

The specimens from young animals which had not reached sexual maturity were taken on the exact day of their indicated age. Tissues from sexually mature animals were obtained on the second day of their sexual cycle and as near as possible to their described age. The material was obtained from swine of several breeds including Landrace, Duroc-Jersey, and Poland China. Gross examination and breeding indicated that all were healthy and normal animals.

The animals were killed by exsanguination and the specimens were obtained at the earliest possible time compatible with the normal abattoir procedure. The ovaries, oviducts, uterus, cervix, and vagina were collected from each animal and the endocrine glands from some. As a rule, four blocks of uterine cross sections were obtained, two from the uterine body and two from the middle part of a uterine horn. One of each of the uterine sections was fixed in 10 per cent formalin and the other in chilled 80 per cent alcohol (modified Gömöri technique).[5] The genital tracts of young animals were fixed, *in toto*, in 10 per cent formalin. The blocks were embedded in paraffin and were sectioned from 4 μ to 8 μ in thickness.

TABLE 1—Number and Age Distribution of Pigs Studied for Uterine Age-Changes

Age	No. of animals	Fixative
1 day	2	F.
2 weeks	2	F.
3 weeks	2	F.A.
4 weeks	2	F.A.
5 weeks	2	F.A.
6 weeks	2	F.A.
7 weeks	2	F.A.
8 weeks	2	F.A.
3 months	2	F.A.
4 months	2	F.A.
5 months	2	F.A.
6 months	2	F.A.
7 months	2	F.A.
8 months	2	F.A.
9 months	3	F.A.
10 months	2	F.A.
12 months	2	F.A.
14 months	1	F.A.
20 months	1	F.A.
33 months	1	F.A.
Total	38	

F = 10 per cent formalin; A = 80 per cent ethyl alcohol.

The histological procedures were as follows: (1) hematoxylin and eosin staining for general orientation; trichrome stains (Masson's and Crossman's)[3] and reticular staining (Wilder)[3] to show the distribution of connective tissue fibers; and (2) measurements were made of the thickness *(a)* of the uterine wall, *(b)* of the endometrium, and *(c)* of the myometrium using an S.V.E. Microbeam* microslide attachment with a 1-inch focal length. The sections were projected to a white screen on which the image was measured with a decimal ruler. There were five sections obtained from each formalin-fixed block. On each section four measurements were made. The given number is the average result of the measurements.

OBSERVATIONS

The observations have been tabulated in the following way: *(a)* proportional changes in the endometrium and myometrium, *(b)* appearance and development of the uterine glands, and *(c)* formation of the uterine mucosal folds.

Proportional Changes in the Endometrium and Myometrium.—The changing proportions of endometrium and myometrium in the uterine wall of the growing

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The animals used in the present study were produced in the swine breeding project conducted cooperatively by the Iowa Agricultural Experiment Station and the U.S. Department of Agriculture, ARS, AHRD, Regional Swine Breeding Laboratory, Ames, Iowa.

The authors thank Dr. Raymond L. Morter, who is now at the College of Veterinary Medicine, Michigan State University, for collection and fixation of the tissues, and to Mr. Joseph T. Morrissey, herdsman at the Regional Swine Breeding Laboratory, Ames, Iowa, for his care and reliability in keeping the records of the ages of the animals.

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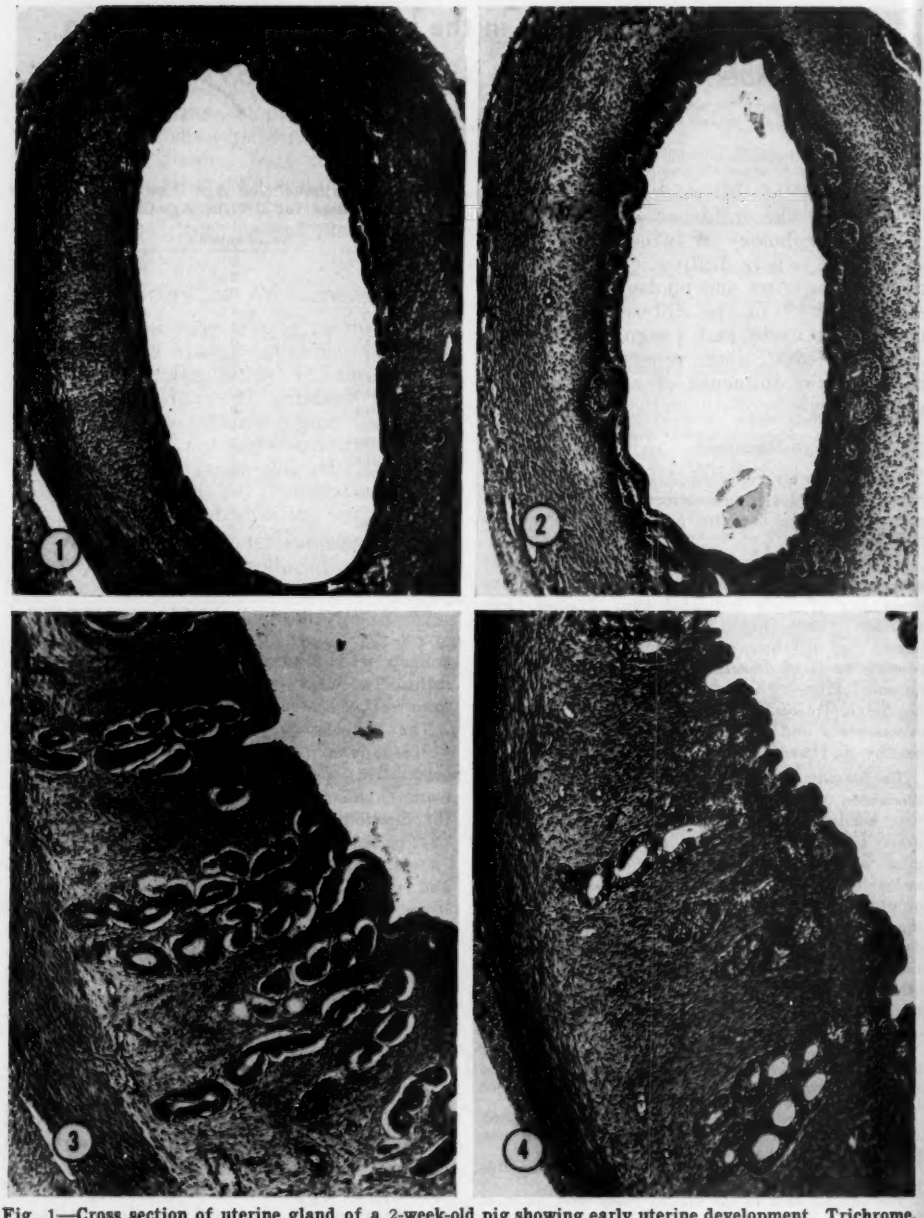
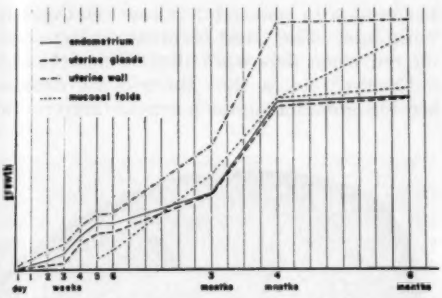


Fig. 1—Cross section of uterine gland of a 2-week-old pig showing early uterine development. Trichrome, Crossman's modification; x 65.

Fig. 2—Section of the wall of the uterine horn of a 3-week-old pig showing uterine glands. Trichrome, Crossman's modification; x 78.

Fig. 3—Section of the uterine body of a 3-month-old pig showing uterine glands penetrating the endometrium. Trichrome, Crossman's modification; x 78.

Fig. 4—Section of the uterine horn of a 5-week-old pig showing endometrial folds. Trichrome, Crossman's modification; x 78.



Graph 1—Linear comparison of uterine growth (table 2).

pig are shown (table 2, graph 1). In the newborn pig, the total thickness of the uterine wall measured 126 μ, and the endometrium amounted to about half of the thickness of the uterine wall (55%). This proportion was found to alter in the growing animals in two ways. First, there was an increase in the thickness of the uterine wall; the 126-μ total wall thickness in the newborn pig gradually increased to its maximum of 5,520 μ at about 6 months of age. From then on, it remained more or less constant, showing only small variations.

The proportions of the different components within the uterine wall, particularly the endometrium and myometrium, altered in the following manner: In the newborn pig, the endometrium represented 55 per cent of the total uterine wall thickness; at 2 weeks of age, this proportion was only 47 per cent; at 3 weeks, it was 62 per cent; at 4 weeks, 77 per cent; and in 5-week-old pigs, it reached 89 per cent of the total thickness of the uterine wall. In the 6-week-old pig, the endometrium was reduced to only 80 per cent in proportion, whereas the myometrium and perimetrium showed an increase in thickness. In the 5-month-old gilt, the myometrium and perimetrium increased further in relative size, reaching maximum development in the six-month-old animal.

Appearance and Development of the Uterine Glands.—Uterine gland appearance and development is shown (table 2; graph 1). In the newborn pig, a few shallow depressions in the uterine epithelium were observable which simulated the appearance of a uterine gland. When pigs were 2 weeks of age, the early glands had comparatively large, funnel-like openings

TABLE 2—Measurements (in Microns) of the Uterine Wall, Glands, Mucosal Folds, and Endometrium of the Pig

Age	Uterine wall (thickness μ)	Endometrium (thickness μ)	Uterine gland (length μ)	Mucosal folds (width μ)
1 day	126	70(55)	.....	.....
2 weeks	420	200(47)	100	.....
3 weeks	546	336(62)	126	.....
4 weeks	926	714(77)	546	.....
5 weeks	1,176	1,050(89)	756	250
6 weeks	1,260	1,008(80)	840	420
3 mo.	2,730	1,680(60)	1,080	2,100
4 mo.	5,460	3,696(69)	3,696	3,780
6 mo.	5,520	3,822(69)	3,822	.....

* Figures in parentheses represent the percentage of total wall thickness.

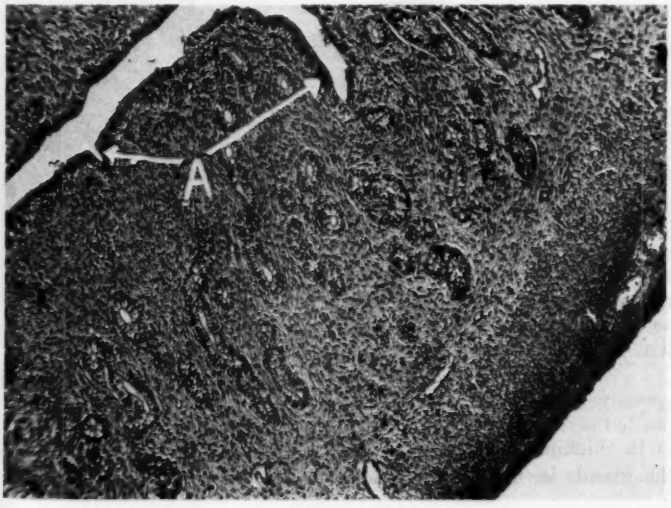


Fig. 5—Section of uterine horn of a 6-week-old pig showing endometrial folds (A). Trichrome, Crossman's modification; x 53.

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which were lined by uterine epithelial cells (fig. 1). In the 3-week-old pigs, the glands penetrated to some depth in the endometrium but, since the endometrium was narrow at this age, the glands never appeared longer than 130 μ (fig. 2).

The gland continued to remain shallow and trailed behind the endometrial thickness until the animal was 3 months old, at which time it penetrated the whole endometrium, reaching the myometrium (fig. 3). From that age on, the depth of the glandular penetration remained at the myometrial border. From the beginning of their growth, the glands penetrated the endometrium like a corkscrew and, in later developmental stages, this tortuosity apparently remained.

Formation of the Uterine Mucosal Folds.—The formation of the uterine mucosal folds is shown (table 2; graph 1). Up to the age of 5 weeks, there was no structure visible in the endometrium of the growing female pig which could be described as a mucosal fold. In the 5-week-old animal, however, small, tonguelike protrusions appeared which projected from the endometrium into the uterine lumen (fig. 4). This type of protrusion, termed mucosal folds in histology textbooks, showed a progressing increase in size. They showed a certain amount of uniformity, which made it possible to measure their approximate size. In the 5-week-old pig, the width of the fold was around 250 μ, which then increased to 420 μ in pigs 6 weeks old (fig. 5). The size changed to 2,100 μ in the three-month old pigs with a corresponding increase in the length (height) of the folds as well (fig. 6). From that age on, the mucosal folds were found to grow with such an irregularity and speed that no further attempts were made to measure them (fig. 7).

DISCUSSION

A number of changes were observed in the uterus of the young pig. One of the foremost was the alteration in the proportion of the endometrium to the myometrium. The endometrium increased from 55 per cent of the uterine wall thickness in the newborn to 69 per cent in the sexually mature animal.

The uterine wall progressively changed from an extremely thin-walled structure of 126 μ to 5,000 μ (5 mm.) in thickness.

The development of the glands in number and size showed a close relationship with age. The most evident changes occurred when pigs were about 3 months old.

Casida [2] states that there is no demonstrable gonadotrophic hormone activity in

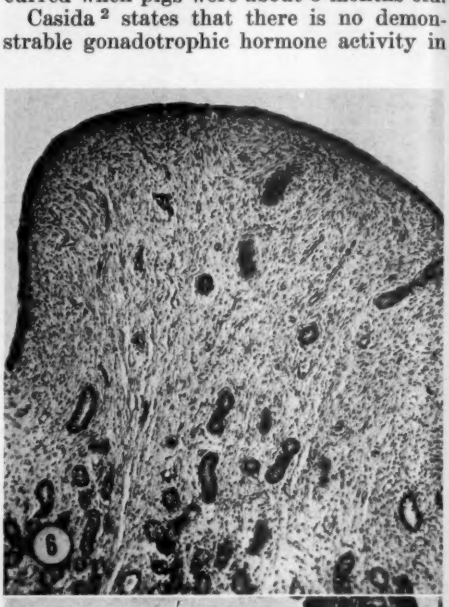

Fig. 6—An endometrial fold in the uterine horn of a 3-month-old pig. H & E stain; x 65.

Fig. 7—Endometrial folds in the uterine horn of a 6-month-old gilt. Masson's Trichrome; x 78.

the genital tract of the young animal. However, it is postulated that from the age of 3 months, the gonadotrophic hormone influences the development of the myometrial layer, the uterine glands, and the mucosal folds.

SUMMARY

A study of the morphological changes which occurred in the genital tract of the growing pig between the ages of 1 day and 6 months determined that:

1) The total thickness of the endometrium increased from 126 μ in the newborn to over 5 mm. (5,520 μ) in the 6-month-old animal.

2) The endometrium changed from 47 per cent of the thickness of the uterine wall in the 2-week-old pig to 69 per cent in the 4-month-old gilt.

3) The uterine glands, which were few in the newborn pig, developed gradually, penetrating the total depth of the endometrium to the myometrial border when pigs were 3 months old.

4) The mucosal folds first appeared in the 5-week-old pig and gradually increased in complexity.

References

[1] Brambell, C. E.: Allantochorionic Differentiations of the Pig Studied Morphologically and Histochemically. Am. J. Anat., *52*, (1933): 397.

[2] Casida, L. E.: Prepubertal Development of the Pig Ovary and Its Relation to Stimulation with Gonadotropic Hormones. Anat. Rec., *61*, (1935): 389.

[3] Conn, J. H., and Darrow, M. A.: Staining Procedures. Biotechnical Publications, Geneva, N.Y., 1948.

[4] Corner, G. W.: Cyclic Changes in the Ovaries and Uterus of the Sow and Their Relation to the Mechanism of Implantation. Contrib. Embryol., *13*, (1921): 119.

[5] Deane, H. W., and Dempsey, E. W.: (Modified Gömöri Technique) The Localization of Phosphatases in the Golgi Region of the Intestinal and Other Epithelial Cells. Anat. Rec., *92*, (1945): 401.

[6] Wisloki, G. B., and Dempsey, E. W.: Histochemical Reactions of the Placenta of the Pig. Am. J. Anat., *78*, (1946): 181.

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Age-Change Studies of the Ovary of the Domesticated Pig

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MANY PAPERS have been written on the ovary of the domestic pig.[1,3,5,6] However, the ovarian histology, as it is affected by the growth and aging of the animal, has not been adequately reported. Therefore, it was thought to be worthwhile to tabulate and describe the age changes observed in the ovary from birth to maturity in the domesticated pig.

MATERIALS AND METHODS

Specimens were collected in 1954 and 1955 from 38 female pigs, representing 16 different age groups from the newborn to those 33 months old (table 1).

The specimens from young animals which had not reached sexual maturity were taken on the exact day of their indicated ages. Tissues from sexually mature animals were obtained on the second day, 30 hours following the first observation of heat, as evidenced by the acceptance of the boar. A definite stage in the sexual cycle was chosen for consistency and accuracy of observations. The animals, which belonged to the Landrace, Duroc-Jersey, and Poland China breeds, were healthy and normal as measured by visible examination and breeding records.

TABLE 1—Number and Age Distribution of Pigs Studied for Age-Change of Ovaries

Age	No. of animals	Fixative
1 day	2	F.
2 weeks	2	F.
3 weeks	2	F.A.
4 weeks	2	F.A.
5 weeks	2	F.A.
6 weeks	2	F.A.
7 weeks	2	F.A.
8 weeks	2	F.A.
3 months	2	F.A.
4 months	2	F.A.
5 months	2	F.A.
6 months	2	F.A.
7 months	2	F.A.
8 months	2	F.A.
9 months	3	F.A.
10 months	2	F.A.
12 months	2	F.A.
14 months	1	F.A.
20 months	1	F.A.
33 months	1	F.A.
Total	38	

F = 10 per cent formalin solution; A = 80 per cent ethyl alcohol.

They were exsanguinated and specimens obtained at the earliest possible time compatible with normal slaughter procedure at Iowa State College abattoir. The ovaries, oviducts, uterus, cervix, and vagina were collected from each animal and the endocrine glands from some. At least one ovary from each animal and tissue samples of the remaining parts of the reproductive tracts were fixed in 10 per cent formalin solution; the other ovary and duplicate sections were fixed in 80 per cent alcohol (modified Gömöri's technique). The genital tracts of the 1-day and 2-week-old animals were fixed *in toto* in 10 per cent formalin solution.

The blocks were embedded in paraffin and sections were made 4 μ to 8 μ in thickness. Staining procedures used were: hematoxylin-eosin staining for general orientation; trichrome stains (Masson's and Crossman's modification of Mallory's[4]) to show the distribution of connective tissue fibers; and methyl-green pyronine (Unna-Pappenheim, Glick[5]) stains to indicate the intracellular proportion of ribonucleic acid as compared with desoxyribonucleic acid.

In addition to histological observations, follicular counts were taken to determine the proportion of follicles in the various developmental stages. Since follicular counting was completed only for the sake of comparative numbers, it was endeavored to select identical cortical areas roughly corresponding to a cross section through the middle of the ovary, in such a way as to form a 90-degree angle with the ovarian longitudinal axis. In these areas, a minimum of 500 follicles were counted in each ovary, using an S.V.E.* microslide attachment to a 2- by 2-inch slide projector.

The observations were tabulated in the following way:

I. Submacroscopic observations.

II. Changes in the parenchyme which in turn has been subdivided into sections dealing with: *(a)* alteration in the germinal epithelium, *(b)* alteration in the follicular constituents of the cortex, and *(c)* changes in follicular development.

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III. Changes in the stroma which has been subdivided into sections dealing with: *(a)* alteration in the cortical and medullary connective tissue and *(b)* appearance of the tunica albuginea.

OBSERVATIONS

SUBMACROSCOPIC OBSERVATIONS

In the newborn pig, the ovary presented a trilobed appearance macroscopically. Microscopic examination showed the three clefts were discernible (fig. 1); however, each lobe presented a cortical and medul-

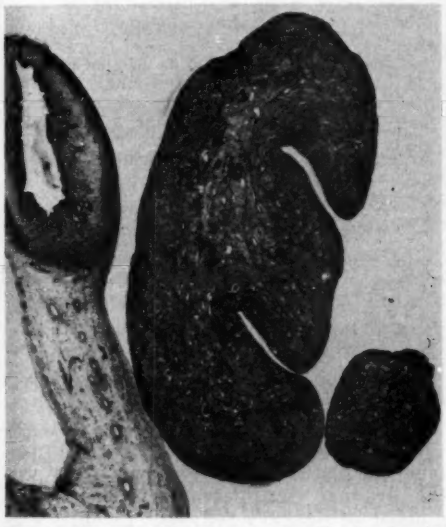

Fig. 1—Ovary of the pig, 2 weeks old, showing its trilobed appearance. H & E stain; x 35.

lary portion (fig. 2). The clefts became less predominate in pigs 5 to 6 weeks old and, in 3-month-old pigs, they had regressed completely. Occasionally, clefts were observed in the ovaries of mature hogs.

CHANGES IN THE PARENCHYMA

Alterations in the Germinal Epithelium.—Two different features connected with the germinal epithelium were considered worth describing: (1) the alterations in the shape of germinal epithelial cells in different parts of the ovary, and (2) the occasional presence of cell clusters in the ovarian surface immediately beneath the germinal epithelium.

1) There were areas where the germinal epithelium appeared to be tall columnar,

Fig. 2—Ovarian cortex of a 3-week-old pig. Notice the lobulation and the densely packed follicles. H & E stain; x 80.

measuring up to 30 μ, while at other places, often next to these areas, it was flat or low cuboidal, measuring only 10 μ (fig. 3). This alteration in the epithelial behavior could be observed from birth to 33 months of age. Neither the hematoxylin-eosin stain nor methyl-green pyronin showed any differ-

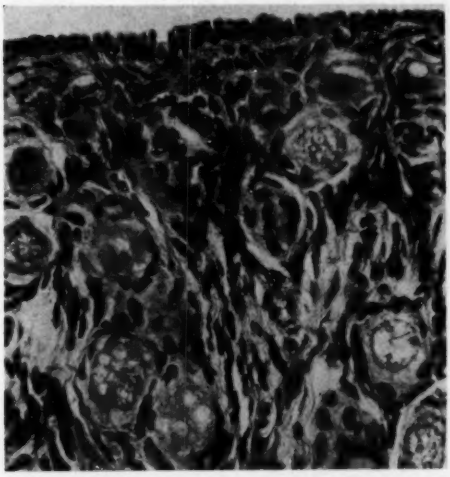

Fig. 3—Section of ovarian cortex of a pig, 6 weeks old, showing low cuboidal to flat germinal epithelium. Trichrome, Crossman's modification; x 435.

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ences in the cytoplasmic staining or nuclear reaction in the tall epithelial cells, when compared with the smaller cells.

2) The position of the cell clusters was not characteristic of any particular age. The cells forming them were round to polyhedral in shape, and occasionally some of them had a clear, unstained cytoplasm (fig. 4). In the sections studied, there was no apparent cell division observed in these islands, and no increased reaction with the methyl-green pyronin staining.

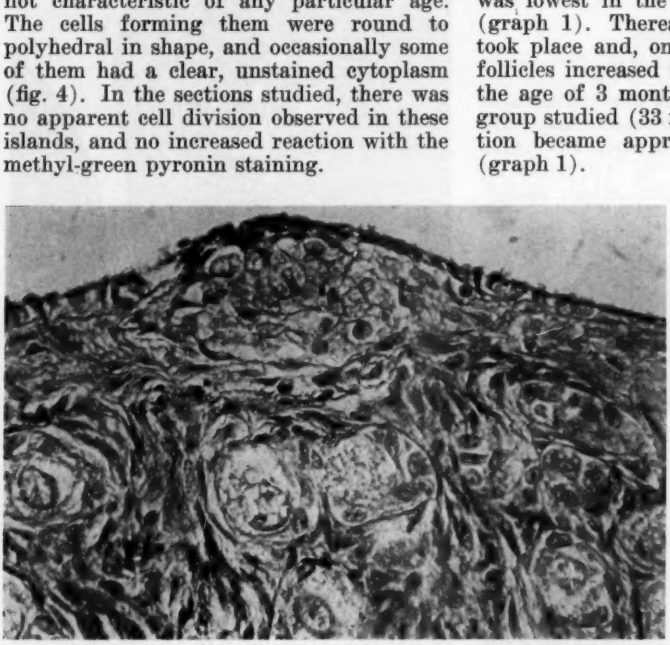
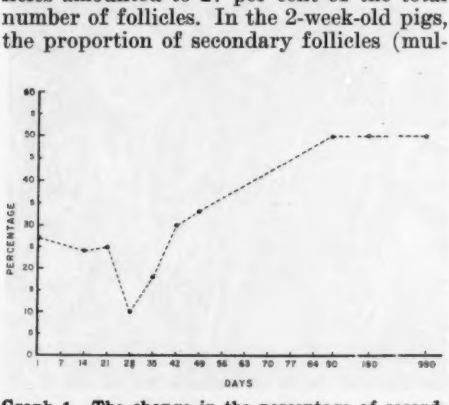


Fig. 4—Section of ovary of a 7-month-old pig showing cell clusters beneath the germinal epithelium. Methyl-green pyronin; x 475.

Alteration in the Follicular Constituents of the Cortex.—At birth, the ovarian cortex was densely packed with small primary follicles (single layers of follicular cells around each ovocyte). The secondary follicles amounted to 27 per cent of the total number of follicles. In the 2-week-old pigs, the proportion of secondary follicles (multiple layers of follicular cells) was 24 per cent of the total. With increase in age, this proportion changed in favor of the primary follicles. The number of secondary follicles was lowest in the 4- and 5-week-old pigs (graph 1). Thereafter, a gradual change took place and, once again, the secondary follicles increased in number so that from the age of 3 months through the last age group studied (33 months old), the proportion became approximately 50 per cent (graph 1).



Graph 1—The change in the percentage of secondary follicles compared with the total count in the ovary of the swine.

The characteristic alteration in the histological picture of the growing ovary was the gradual disappearance of the large number of primary follicles. In older animals, their place was taken by solitary follicles. The first follicles in which liquor formation occurred were seen at 7 weeks of age. The first fully developed follicular cavity was observed in an animal 3 months old (fig. 5).

When ovarian ages are judged solely on the basis of follicular components, three periods can be differentiated: (1) the early, prepuberal ovarian period, characterized by the presence of large numbers of primary follicles (fig. 6), with liquor production not more progressed than in the Call-Exner body stage, and low amounts of connective tissue (ovaries of pigs up to 8 weeks old); (2) the next ovarian period, characterized by large vesicular follicles and the comparatively low amount of connective tissue (pigs 3 to 7 months old); and (3) the final period, characterized by an increased amount of connective tissue and a com-

paratively low proportion of follicular components (gilts over 8 months old).

Changes in Follicular Development.—Multinuclear Ova.—Most frequently, two cells were affected, although three, four, and, occasionally, five were also observed within the same follicle. The individual cytoplasmic boundary of the ovocytes was completely absent and only on the periphery was a thin follicular membrane visible. The nuclei were never coalescent, but were always completely separated from one another.

In the relationship of age to multinuclear ova, the following alterations were observed: *(a)* the ovaries of the newborn pigs presented the maximum number of multinuclear ova(fig. 7), almost equally distributed throughout the whole cortical area; *(b)* at the end of the second week there were still many present, although their number was decreasing (no counts were made) and only a few were present in the outer part of the cortex; *(c)* the number of multinuclear ova was considerably lower at 3 weeks of age and only a few were present at 4 weeks, those present being found only in the inner cortical area; and *(d)* at no age, from 5 weeks onward through the whole series studied, were multinuclear ova observed again.

Polyovular Follicles.—The polyovular follicles, as a rule, were round. The numbers of ovocytes recovered in large polyovular follicles varied from two to ten or more, but follicles generally contained three to six ovocytes (fig. 8). Large numbers of polyovular follicles were present in the newborn pigs, but their number decreased throughout the first 4 weeks of age. After pigs were approximately 5 weeks old, practically no polynuclear follicles were observed.

Follicular Atrophy.—Since detailed descriptions of follicular atrophy have been published, it will be considered in this paper only as a phenomenon which affects the ovaries of gilts in a different way than the ovaries of sows.

Atrophy was found to be present in specimens from the youngest (fig. 9) to the oldest pigs. In the ovaries of pigs under 5 weeks of age, there were remarkably large numbers of primary follicles and ovocytes degenerating. In pigs over 5 weeks old, the number of atrophying ovocytes and primary follicles gradually decreased. In sexually mature ovaries, only low numbers of degenerating ovocytes were observed.

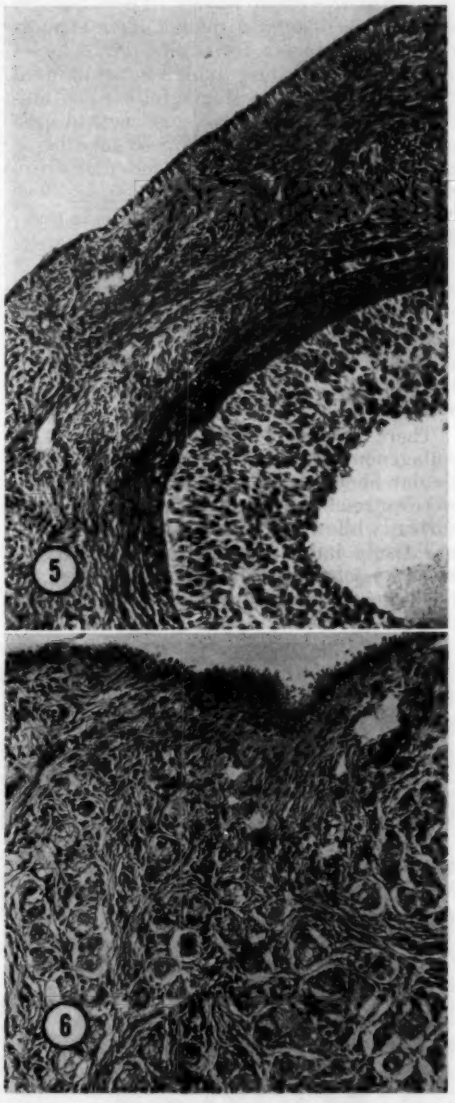


Fig. 5—Ovarian cortex of a 3-month-old gilt. Silver stain; x 85.

Fig. 6—Ovarian cortex of a 2-week-old pig showing the comparatively large number of follicles and scant connective tissue fibers. H & E stain, x 170.

CHANGES IN THE STROMA

Alteration in the Cortical and Medullary Connective Tissue.—In the ovaries of swine less than 3 months old, a reticular, fiber-rich area was found in the medulla, which contained the blood vessels. There was an area where scanty reticular fiber strands divided the cortex into follicular colonies which, in turn, were divided by individual reticular fibers into single follicle-containing units. This reticular-poor cortical area gradually changed until, by 33 months of age, it was at least as rich in reticular fibers as the medulla.

The direction of reticular fibers in the cortex also underwent considerable change. In young animals, the fibers were directed radially from the medulla to the cortex or rather toward the germinal epithelium, whereas in animals over 4 months of age, fibers were seen to be running parallel with the germinal epithelium and the ovarian surface.

There was an increase in the amount of collagenous fibers similar to that of the reticular fibers. In the 2-week-old pigs, there were extremely few collagenous fibers in the cortex, while the medulla contained a comparatively large amount, in which all the blood vessels were embedded. However, in the 4-month-old pigs there was a well-established layer of connective tissue between the germinal epithelium and the follicles.

Appearance of the Tunica Albuginea.—The development of a tunica albuginea is gradual, and its thickness is never impressive. Only after the pig is 3 months old, when the thin threads of collagenous and reticular fibers have accumulated into a more easily distinguishable layer, can one speak of a tunica albuginea. In the sections studied, its thickness was never beyond 30 μ.

DISCUSSION

The material was not suitable for serial sectioning, but it appeared remarkable that, even in large ovaries, germinal epithelial islands could be found beneath the germinal epithelium. These islands were never close to one another. Raps [13] described similar structures in the ovary of the dog.

From the viewpoint of the alternating height of the germinal epithelium, the sow shows some likeness to the dog. The difference between the two species is that the columnar epithelial cells in the bitch appeared only at a certain age, from which it was concluded that they can be regarded as a potential source of ovocytes, acting only when the previous store of ovocytes is exhusted.[13] In the ovary of the swine, however, it was a constant observation, even at

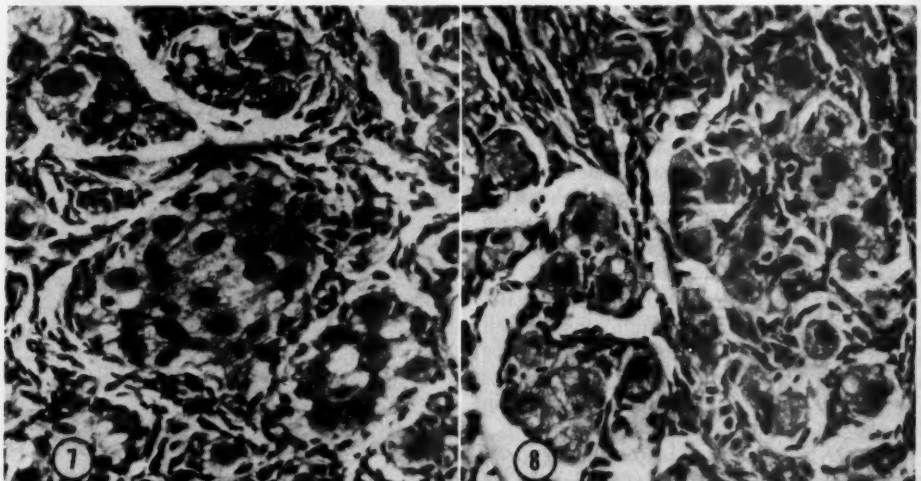


Fig. 7—Ovary of a newborn pig showing mutinuclear ova. H & E stain; x 410.

Fig. 8—Section of an ovary from a 2-week-old pig showing the polyovular follicles in the process of multinuclear ova formation. H & E stain; x 410.

ages when the ovary was predominantly filled with primary follicles. It was hoped that with Unna-Pappenheim staining a difference could be detected between the ribonucleic and desoxyribonucleic acid content of these cells, thus throwing some light on potential future ovocytes. However, no differential characteristics were observed.

Therefore, it is difficult to explain the possible function of the germinal epithelial islands. The simplest explanation would be that they are the precursors of young ovocytes and follicles. However, this would be a contradiction to the results of Mandl and Zuckerman,[11,12] who maintain that in the rat, at least, there is no new formation of follicles after birth.

Another interesting question is that of hormonal influence on the ovaries of the young pig. Casida [3] injected gonadotrophic hormones into prepubertal gilts and was unable to induce ovulation in any of the sexually immature animals. Consequently, he arrived at the conclusion that the ovaries of the young prepubertal gilt are nonresponsive to gonadotrophic hormones. This conclusion is of interest since, in this study, it has been observed that extensive follicular atrophy occurs for a long time before the gilts reach ovulating age.

The gradual alteration in the follicular constituents of the ovary and the gradual increase in the amount of connective tissue are all similar to the features encountered in the ovary of the dog.[13]

With regard to the presence of the multinuclear ovocytes and polyovular follicles in the present series of ovaries examined, they were found only in the ovaries of the very young animals. Their number gradually diminished, until practically none were present after the age of 5 weeks.

Bacsich [2] observed similar structures in the newborn human female. Harrison [10] and Hadek [9] reported similar structures in the ovaries of goats and sheep with regular estrous cycles, but only during the proestrous or estrous periods. Therefore, these structures are thought of as freaks produced by the somewhat excessive activity of the pituitary's gonadotrophic hormone. In the ovary of the neonatal pig it seems that, due to the gonadotrophic activity of the chorion, there is a large-scale development of the multinuclear ovocytes and polyovular follicles which completely disappear after birth in the absence of the hormonal activity. This may explain why the cortex of the ovary in the young pig is so full of ovocytes and follicles. Later, the number of the ovocytes and follicles decreases. In this respect, sows seem to differ from ewes. The ovary of the sexually mature sheep contains quite a large number of polynuclear ovocytes and polyovular follicles, which develop in pre-estrus and atrophy in metestrus, whereas practically none were visible in the sexually mature pig.

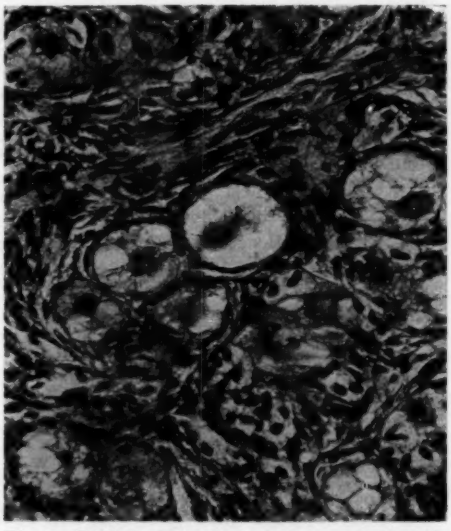


Fig. 9—Follicular atrophy in an ovary of a 3-week-old pig. Notice disintegration of ovocyte cytoplasm. Masson's trichrome; x 395.

SUMMARY

In a morphological study of swine ovaries, specimens from 38 female swine, ranging from 1 day to 33 months of age, were studied. The changes observed can be summarized as follows:

1) The presence of germinal epithelial cell clusters appeared to be a regular feature visible in practically every ovary from birth to 33 months of age.

2) Extremely little cortical stroma was present in the ovaries of the newborn pig.

3) At birth, the entire ovarian cortex and a large part of the medulla was occupied by primary follicles.

4) In the growing pig, the number of primary follicles increased up to 4 weeks of age; thereafter, they appeared to decrease until equilibrium was reached at 6 months of age.

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5) The first vesicular follicles appeared at 5 to 6 weeks of age.

6) Multinuclear ova and polyovular follicles were found during the first 5 weeks of extrauterine life and were thought to be closely associated with the chorionic gonadotrophic secretion.

7) During the first 6 weeks of extrauterine life, many follicles became atrophied.

8) Further statistical studies should be made to evaluate the true significance of the relative proportions given.

9) The development of the tunica albuginea was gradual, being first distinguishable at 3 months of age.

References

[1] Amman, K.: Histologie des Schweine-eierstockes unter besonderer Beruksichtigung des Ovarialzyklus. Dissertation, Universitat Zurich, 1936.

[2] Bacsich, P.: Some Observations on Near Term Human Foetal Ovaries. J. Endocrinol., *5*, (1948): 141.

[3] Casida, L. E.: Prepubertal Development of the Pig Ovary and Its Relation to Stimulation with Gonadotrophic Hormones. Anat. Rec., *61*, (1935): 389.

[4] Conn, H. J., and Darrow, M. A.: Staining Procedures. Biotechnical Publications, Geneva, N.Y., 1946.

[5] Corner, G. W., and Amsbaugh, A. E.: Cyclic Changes in the Ovaries and Uterus of the Sow and Their Relation to the Mechanisms of Implantation. Carneg. Contrib. Embryol., *13*, (1921): 117.

[6] Corner, G. W., and Amsbaugh, A. E.: Oestrus and Ovulation in Swine. Anat. Rec., *12*, (1917): 287.

[7] Deane, H. W., and Dempsey, E. W.: (Modified Gömöri Technique) The Localization of Phosphatases in the Golgi Region of the Intestinal and Other Epithelial Cells. Anat. Rec., *92*, (1945): 401–417.

[8] Glick, D.: Techniques of Histo- and Cyto-Chemistry. Interscience Publishers, New York, N.Y., 1949.

[9] Hadek, R.: Morphological and Histochemical Study on the Ovary of the Sheep. Am. J. Vet. Res., *19*, (Oct., 1958): 873.

[10] Harrison, R. T.: The Changes Occurring in the Ovary of the Goat During Oestrus Cycle and Early Pregnancy. J. Anat., *88*, (1954): 21.

[11] Mandl, A. M., and Zuckerman, S.: The Growth of the Ovocyte and Follicle in the Adult Rat. J. Endocrinol., *8*, (1952a): 126.

[12] Mandl, A. M., and Zuckerman, S.: Cyclical Changes in the Number of Medium and Large Follicles in the Adult Rat Ovary. J. Endocrinol., *8*, (1952b): 341.

[13] Raps, G.: The Development of the Dog Ovary from Birth to 6 Months of Age. Am. J. Vet. Res., *9*, (Jan., 1948): 61.

The Effects of Infecting Newborn Pigs with Transmissible Gastroenteritis Virus

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TRANSMISSIBLE GASTROENTERITIS (TGE) of baby pigs was first described by Doyle and Hutchings.[5] The clinical signs were vomiting, diarrhea, dehydration, loss of body weight, and development of a rough hair coat. Mortality can approach 100 per cent in pigs 1 week of age or less.[5, 8, 15]

Several investigations have revealed changes in blood glucose, blood urea, and nonprotein nitrogen in baby pigs infected with TGE. Reber and Whitehair[10] found pigs 26 days of age infected with TGE had a lower blood glucose concentration than noninfected pigs. Whitehair *et al.*[16] found a steady decline of blood glucose with a marked increase of blood urea nitrogen and nonprotein nitrogen. Bay *et al.*[1] did not observe differences in the level of blood glucose of TGE-infected and noninfected baby pigs.

This is a report of an investigation to determine the levels of glucose, nonprotein, and urea nitrogen in the blood of pigs, 1 to 5 days old, that have been infected experimentally with TGE. It was also designed to determine if the weights of several organs of TGE-infected pigs, when compared with those of noninfected pigs, could be correlated with TGE virus titer as determined by Lee *et al.*[8]

MATERIALS AND METHODS

Litters of pigs from 10 Hampshire sows were used in this study. Five of the litters (40 pigs) were infected with TGE, and the other five litters (36) pigs were not infected. The newborn pigs to be infected were each given orally, by syringe, about 1 ml. of a supernatant fluid from a saline solution suspension of a ground intestine taken from a TGE-infected pig. The supernatant fluid had been stored at −13 C. Two o'clock in the afternoon was designated as 0 time; at this time, the pigs were not more than 24 hours old. One pig from each litter was killed at 0 time, 6 hours later, and at 12-hour intervals thereafter. Baby pigs were selected at random at these intervals and anesthetized with chloroform. Blood samples were obtained by heart puncture. Potassium oxalate was used as an anticoagulant.

All pigs were killed by exsanguination and their livers, spleens, lungs, and kidneys were removed and weighed. The esophagus was severed as close to the stomach as possible, and the remaining portion of the small and large intestines were freed from their attachments. The stomach contents were removed and the emptied stomach, intestines, and their contents weighed together. The emptied stomach and the intestine and its contents will be referred to as the gastrointestinal (G.I.) tract. A piece of liver weighing 0.2 to 0.5 Gm. was used for glycogen determinations. The liver samples from pigs of seven litters were placed in 30 per cent KOH. The liver samples of the remaining litters were either frozen by means of an alcohol-dry-ice mixture or placed in a freezer at −13 C. Liver glycogen was determined by the method of Hansen *et al.*[7] Blood glucose determinations were made by using a modified Folin-Wu method.[14] Hemoglobin was determined by a modification of the cyanmethemoglobin method of Crosby *et al.*,[3] and blood nonprotein nitrogen and urea nitrogen were determined by the method described by Chaney and Lamar.[2]

All of the determinations for blood components and liver glycogen of the noninfected and TGE-infected pigs were compared statistically from 0 time to the fifty-fourth hour of this experiment. The body and organ weights were compared statistically for the same interval of time, both as organ weights *per se* and as organ weights adjusted for body weight.

RESULTS AND DISCUSSION

Severe diarrhea was observed in all pigs infected with TGE virus within 24 hours after exposure. The diarrhea persisted for the duration of the experiment. The infected pigs became lethargic, gaunt, and appeared dehydrated, as evidenced by loose skin on the thighs and abdomen. However, there were no deaths in the group of infected pigs. No clinical signs of TGE or any other diseases were seen in the noninfected pigs.

From the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine, and the Agricultural Experiment Station, University of Illinois, Urbana, as a collaborator under the North Central Regional Agricultural Experiment Station cooperative research project "Death Losses in Young Pigs."

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TABLE 1—Average Body and Organ Weights (Gm.) of Noninfected and Transmissible Gastroenteritis-Infected Pigs

Time (hr.)	Body weight		Lung		G.I. tract		Kidney		Spleen	
	N	TGE	N	TGE	N	TGE	N	TGE	N	TGE
0	1,147(5)*	1,489(5)	19.2(5)	24.4(5)	77.8(5)	80.8(5)	9.2(5)	11.8(5)	1.4(5)	1.6(5)
6	1,332(5)	1,254(5)	20.0(5)	20.2(5)	91.2(5)	78.2(5)	9.8(5)	10.0(5)	1.4(5)	1.0(5)
18	1,318(5)	1,633(5)	20.0(5)	27.2(5)	87.6(5)	106.4(5)	10.6(5)	13.4(5)	1.8(5)	2.2(5)
30	1,422(5)	1,297(5)	22.0(5)	21.2(5)	94.6(5)	70.2(5)	9.8(5)	11.0(5)	3.6(5)	1.6(5)
42	1,474(5)	1,371(5)	24.0(5)	23.2(5)	91.6(5)	97.2(5)	11.2(5)	11.9(5)	1.6(5)	2.4(5)
54	1,565(5)	1,456(5)	25.6(5)	26.4(5)	100.0(5)	113.0(5)	11.2(5)	12.0(5)	2.4(5)	1.8(5)
66	1,847(2)	1,451(5)	28.5(2)	27.8(5)	110.5(2)	108.0(5)	14.0(2)	12.6(2)	2.5(2)	1.6(5)
78	1,572(2)	1,057(3)	23.0(2)	20.2(2)	107.0(2)	70.7(4)	12.5(2)	8.8(4)	3.0(2)	1.0(4)
90	1,971(2)	1,052(2)	31.5(2)	16.0(2)	131.0(2)	47.5(2)	15.0(2)	8.0(2)	4.0(2)	1.0(2)

N = noninfected pigs; TGE = pigs infected with transmissible gastroenteritis; G.I. = gastrointestinal tract.
* Numbers in parentheses represent the total number of determinations.

The average body and organ weights of noninfected and TGE-infected pigs from 0 time to the ninetieth hour are tabulated (table 1). The individual body weights of the pigs were quite variable within both noninfected and infected litters. There was no significant difference between the body weights of noninfected and infected pigs from 0 time through the fifty-fourth hour. After hour 54, the body weights of infected pigs appeared to be much lower than those of the noninfected pigs (table 1). However, the small number of pigs used beyond hour 54 precluded statistical evaluation. The loss of body weight observed in this study confirms other work.[5, 8, 10, 15]

No significant difference was observed in G.I. tract, lung, liver spleen, or kidney weights *per se* between noninfected and infected pigs through hour 54. The G.I.-tract weights of the infected pigs were found to be significantly greater ($P = 0.05$) than those of the noninfected pigs from 0 through the hour 54 when the G.I. tract weights were adjusted to equal body weight.

The increase was probably due to edema of the mucosa and submucosa. It was not due to increased luminal content because, at necropsy, infected pigs had very little luminal content. Edema of the intestinal mucosa of TGE-infected pigs has been observed by Bay *et al.*[1] Lee *et al.*[8] reported that the intestine and kidney had the highest titers of TGE virus. There appears to be a correlation of high titer of TGE virus and the occurrence of edema, as reflected in the significant increase in G.I. tract weight, but no such correlation appears with respect to the kidney.

Lung weights were not significantly different between infected and noninfected pigs when adjusted to equal body weight. The lungs of both the noninfected and infected pigs increased in weight at about twice the rate the body weight increased through the fifty-fourth hour of this experiment.

The average body weight of 5 infected pigs killed at hour 78 and 90 was 1,055 Gm. This low body weight is considered to be due to the effects of TGE. Therefore, the lung, G.I. tract, kidney, and spleen weights might be expected to be much lower in pigs infected with TGE than noninfected pigs of the same age. This could be verified by another experiment in which a sufficient number of pigs would be examined to permit statistical evaluation.

The average values of blood total protein, hemoglobin, urea nitrogen, nonprotein nitrogen, and glucose and liver glycogen content of noninfected and infected pigs from 0 to hour 90 of this experiment are shown (table 2). The increases in the total blood protein and hemoglobin, along with the losses of body weight, severe diarrhea, and the physical appearance of gauntness, indicated that the pigs infected with TGE virus were dehydrated after the fifty-fourth hour of the experiment. No significant difference for total blood protein or hemoglobin was found between infected and noninfected pigs through the fifty-fourth hour. Particular emphasis was given to the blood glucose and liver glycogen content of the infected pigs of this study because of their possible relation to hypoglycemia.

A fatal condition in newborn pigs was reported by Graham *et al.*,[6] in which hypoglycemia seemed to be the most striking characteristic. Sampson *et al.*[12] compared the blood glucose concentration and liver glycogen content of pigs less than 96 hours old with clinical cases of hypoglycemia. Blood glucose concentrations ranged from 3 to 61 mg./100 ml. in the clinical cases and from 9 to 43 mg./100 ml. in experi-

mentally fasted, newborn pigs. The liver glycogen content in both clinical and experimentally fasted pigs was lower than the limits of the method of determination.

In this study, there was a marked variation in the blood glucose of infected pigs which varied from 100 to 231 mg./100 ml. Noninfected baby pigs were able to maintain a level of blood glucose within a restricted range of 117 to 155 mg./100 ml. There was no significant difference between the blood glucose of infected and noninfected pigs from hour 0 to 54 of this experiment. Sampson and Newton [18] reported hypoglycemic coma in baby pigs in which the blood glucose level was 40 mg./100 ml. Pigs, 4 weeks old, infected with TGE had an average blood glucose concentration of 63 mg./100 ml.[10] This low concentration of 63 mg./100 ml. was not considered as indicating hypoglycemia. In this experiment, there was no indication of any hypoglycemia associated with TGE.

The average liver glycogen content of infected baby pigs decreased after the eighteenth hour following infection and remained lower than values obtained from noninfected pigs. There was no statistically significant difference between liver glycogen contents of infected and noninfected pigs from hour 0 to 54. Only traces of glycogen could be found in the livers of infected pigs at hours 78 to 90, while noninfected pigs had the highest liver glycogen content encountered during the experiment (2.69%). The average liver glycogen content of noninfected pigs was 1.4 per cent, as compared with 2.7 per cent reported by Morrill [9] for suckling pigs 15 to 150 hours old. Blood glucose levels ranged from 75 to 145 mg./100 ml.

These data indicate that hypoglycemia is not associated with TGE infection, and that TGE infection appears to inhibit glycogenesis in some way. This is suggested by the concurrence of glycogen-depleted livers with adequate or elevated blood glucose levels. The blood glucose may be maintained in TGE-infected pigs by breakdown of protein, which may cause a progressive rise of both blood urea nitrogen and nonprotein nitrogen ($P<0.001$). The linear increase of blood nonprotein and urea nitrogen in infected pigs from 0 to 54 hours may be due largely to endogenous protein breakdown, because dietary intake of protein is known to be low. The stomachs of

TABLE 2—Average Values of Blood Analyses and Liver Glycogen Content of Noninfected Transmissible Gastroenteritis-Infected Baby Pigs

Time (hr.)	Blood glucose (mg./100 ml.)		Blood total protein (Gm./100 ml.)		Blood nonprotein nitrogen (mg./100 ml.)		Blood urea nitrogen (mg./100 ml.)		Hemoglobin (Gm./100 ml.)		Liver glycogen (%)	
	N	TGE	N	TGE	N	TGE	N	TGE	N	TGE	N	TGE
0	125(5)*	139(5)	4.00(5)	4.39(5)	39.0(5)	32.0(3)	27.0(5)	19.0(5)	11.1(4)	10.4(4)	1.30(5)	1.31(5)
6	142(5)	153(5)	5.47(5)	5.23(5)	48.6(5)	69.5(4)	22.6(5)	29.8(5)	10.4(4)	11.6(4)	1.41(5)	1.28(5)
18	121(4)	232(4)	5.62(5)	5.47(4)	46.0(5)	73.2(4)	29.4(5)	23.6(5)	10.7(4)	10.9(2)	0.27(5)	1.55(5)
30	138(5)	100(5)	5.59(2)	5.61(5)	53.4(5)	60.8(5)	28.6(5)	24.2(5)	10.3(4)	9.7(3)	0.60(5)	0.25(5)
42	117(5)	129(5)	4.74(4)	5.61(5)	35.0(5)	71.8(5)	19.4(5)	36.4(5)	9.8(4)	10.1(4)	1.17(5)	0.92(5)
54	131(5)	176(5)	4.85(5)	6.22(5)	37.6(5)	84.8(5)	17.4(5)	50.0(5)	10.8(3)	12.5(3)	1.13(5)	0.38(5)
66	146(2)	128(5)	4.75(2)	6.43(5)	38.5(2)	95.2(5)	.....	.....	12.0(2)	13.3(4)	1.74(2)	0.29(5)
78	114(1)	151(4)	4.54(2)	5.88(3)	35.0(2)	151.8(4)	.....	.....	10.1(2)	12.7(4)	2.28(2)	0.05(3)
90	149(1)	202(2)	5.95(2)	5.85(1)	51.0(2)	240.0(1)	.....	.....	10.9(2)	12.8(2)	2.69(2)	0.14(2)

N = noninfected pigs; TGE = pigs infected with transmissible gastro-enteritis.
* Numbers in parentheses are the total number of determinations.

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infected pigs usually do not contain as much milk as those of the controls. This may be accounted for by emesis which frequently occurs immediately after suckling. The accompanying diarrhea leads also to a marked decrease of foodstuff utilization. High blood urea and nonprotein nitrogen can not be excluded as a reflection of kidney impairment, since Doyle [4] found that the kidneys of infected pigs showed signs of degeneration with urate retention. Until the extent of physiological derangement of the kidney as affected by TGE is determined, the significance of elevated blood urea and nonprotein nitrogen can not be fully evaluated.

SUMMARY

In a study of the effect of transmissible gastroenteritis on suckling newborn Hampshire pigs, no significant difference was found in the organ and total body weights between infected and noninfected baby pigs up to 54 hours following infection.

Total body and organ weights in general were lower in the infected pigs from 54 to 90 hours after infection, probably because of vomiting, diarrhea, and decreased milk consumption. The weight of stomach tissue plus intestinal tract and its contents was higher for infected pigs than for noninfected pigs when adjusted to equal body weight, and is believed to be due to submucosal and mucosal edema.

The total blood protein and hemoglobin of infected pigs were higher than those of the noninfected pigs, which probably reflects dehydration. Blood glucose varied considerably more in infected pigs than in the noninfected pigs, but there was no definite trend toward a sustained hypoglycemia, in spite of the fact that liver glycogen decreased to practically zero. The sustained blood glucose of infected pigs was believed to be largely due to endogenous protein breakdown, which was correlated with higher blood urea and nonprotein nitrogen. Damage to kidney function may also account for the sustained and elevated blood urea and nonprotein nitrogen of infected pigs.

References

[1] Bay, W. W., Hutchings, L. M., Doyle, L. P., and Bunnell, D. E.: Transmissible Gastroenteritis in Baby Pigs. J.A.V.M.A., *115*, (Oct., 1949): 245–248.

[2] Chaney, A. L., and Lamar, I. B.: Clinical Analyses with the Beckman B and DU Spectrophotometer. Beckman Division, Beckman Instruments, Inc., Fullerton, Calif.

[3] Crosby, W. H., Munn, J. I., and Furth, F. W.: Standardizing a Method for Clinical Hemoglobinometry. U.S. Armed Forces Med. J., *5*, (1954): 693–703.

[4] Doyle, L. P.: Some Important Swine Diseases. Canad. J. Comp. Med. & Vet. Sci., *14*, (1950): 225–229.

[5] Doyle, L. P., and Hutchings, L. H.: A Transmissible Gastroenteritis in Pigs. J.A.V.M.A., *108*, (April, 1946): 257–259.

[6] Graham, R., Sampson, J., and Hester, H. R.: Studies on Baby Pig Mortality. I. Acute Hypoglycemia in Newly Born Pigs (so-called Baby Pig Disease). Proc. Soc. Exptl. Biol. & Med., *47*, (1941): 338–339.

[7] Hanson, R. G., Ruther, W. J., and Craine, E. M.: A Nephelometric Method for the Determination of Glycogen. J. Biol. Chem., *195*, (1952): 127–132.

[8] Lee, K. M., Moro, M., and Baker, J. A.: Transmissible Gastroenteritis in Pigs. Am. J. Vet. Res., *15*, (July, 1954): 364–372.

[9] Morrill, C. C.: Studies on Baby Pig Mortality. VIII. Clinical Observations on the Newborn Pig with Special Reference to Hypoglycemia. Am. J. Vet. Res., *13*, (April, 1952): 164–170.

[10] Reber, E. F., and Whitehair, C. K.: The Effect of Transmissible Gastroenteritis on the Metabolism of Baby Pigs. Am. J. Vet. Res., *16*, (Jan., 1955): 116–119.

[11] Roe, C. K. H.: Observations on Experimental Transmissible Gastroenteritis of Pigs. M.S. thesis, University of Illinois, Urbana, 1953.

[12] Sampson, J., Hester, H. R., and Graham, R.: Studies on Baby Pig Mortality. II. Further Observations on Acute Hypoglycemia in Newly Born Pigs (so-called Baby Pig Disease). J.A.V.M.A., *100*, (Jan., 1942): 33–37.

[13] Sampson, J., and Newton, W. C.: Studies on Baby Pig Mortality. VII. The Effectiveness of Certain Sugars Other than Glucose in Alleviating Hypoglycemia Coma in Fasting Newborn Pigs. Cornell Vet., *41*, (1954): 377–381.

[14] Todd, J. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods. A Working Manual of Clinical Pathology. 11th ed. W. B. Saunders Co., Philadelphia, 1948.

[15] Whitehair, C. K., Grummer, R. H., Phillips, P. H., Bohstedt, G., and McNutt, S. H.: Gastroenteritis in Pigs. Cornell Vet., *38*, (1948): 24–29.

[16] Whitehair, C. K., Watt, A. B., and Ross, O. B.: Urea, N.P.N., and Blood Sugar Values of Baby Pigs Exposed to Transmissible Gastroenteritis. J. Anim. Sci., *9*, (1950): 672.

Ionographic Studies of Rabbits Infected with *Eimeria Stiedae*

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SERUM PROTEIN PATTERNS from animals with liver disease are usually characterized by an increase in both the β- and γ-globulin fractions.[1] In rats moderately infected with *Cysticercus fasciolaris*, an increase in the β-globulin component was interpreted as evidence of liver damage.[4] An elevation of the γ-globulin component of serum protein is characteristic of some protozoan infections, but Comer [3] failed to observe such a change in dogs with amebic dysentery.

Kunkel [5] found an increase in β-lipoprotein and a disappearance of the lipoprotein fraction in human patients suffering from biliary cirrhosis. This result could be secondary to other factors, since Bossak [2] obtained similar results in rabbits injected with cortisone.

The present study was initiated to ascertain the degree of liver damage in sublethal liver coccidiosis of the rabbit and to see if an elevation of β-lipoprotein and γ-globulin occurred in this condition.

MATERIALS AND METHODS

Crossbred New Zealand white rabbits were obtained, in litters, from a local dealer. Fecal samples indicated that these animals were free of liver coccidiosis. When the rabbits were approximately 40 days old, one half of the animals of each litter were inoculated with sublethal doses (approx. 150,000) of sporulated *Eimeria stiedae* oocysts. Blood samples were obtained by cardiac puncture at 3, 6, 10, 13, 17, 20, and 24 days after inoculation of the host. Following cardiac puncture, animals were killed for metabolic and histological tissue studies. Two animals, a control and an infected litter mate, were exsanguinated at each interval; a complete set of intervals constituted one replicate. Three replicates were carried out, using a total of 42 rabbits.

Initially, it was intended to use frozen serum samples for ionography, but a review of the literature [7, 10] indicated the need for some caution in this regard. Therefore, an experiment was carried out to determine the effect of aging and freezing on the electrophoretic pattern. There appeared to be an increase in serum albumin percentage with age, coinciding with a decrease in α-globulin while the β- and γ-globulins were unaffected. The number of freeze-thaw cycles had no effect except a slight decrease in serum albumin after the first cycle. In the experiments reported in this investigation, fresh serum was used for ionography.

Protein and lipoprotein determinations were made in duplicate, using Whatman 3 mm. paper in a Durrum type cell* with a veronal buffer having an ionic strength of 0.075 and a pH of 8.60. Separations were accomplished in 16 hours at 5 ma. per cell. Ionographs were stained with bromphenol blue, rinsed in acetic acid, and dried for protein determinations.[6] Total protein was determined in duplicate using micro-Kjeldahl procedure. Two methods were used for lipoprotein visualization. In the second replicate, the strips were stained with oil red O.[9] In the third replicate, the serum was prestained with acetylated Sudan black B.[8] No lipoprotein determinations were made on the first replicate. For quantitation of the fractions, graphs were prepared using an integrating densitometer.†

RESULTS AND DISCUSSION

The mean values and statistical summary for the protein fractions (table 1) and the changes in γ- and β-globulin during the course of infection (graph 1) indicate that the major difference seen at the times chosen for sampling was an increase in β-globulin at the thirteenth day after infection. There was some evidence of increased γ- and β-globulins early in the disease, as observed three days after infection. There was a slight, variable increase with age in the serum albumin of the control animals. The infected animals experienced little change in serum albumin levels.

Three lipoprotein fractions were routinely found, as previously reported by Bossak.[2] However, as two of these fractions (graph 2) have similar mobilities, their percentages are combined (table 2). Although the two staining methods used gave comparable results, the acetylated Sudan black B method [8] was preferable. This lat-

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* Spinco Model R Paper Electrophoresis Cell, manufactured by Beckman Instruments, Inc., Spinco Division, Belmont, Calif.

† Spinco Model R Analytrol, manufactured by Beckman Instruments, Inc., Spinco Division, Belmont, Calif.

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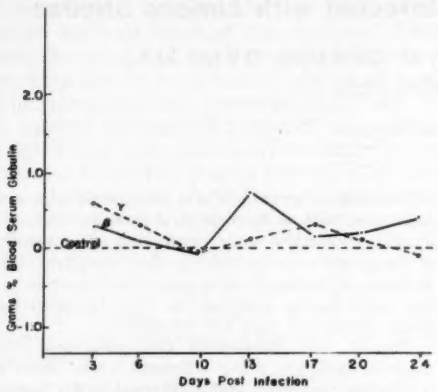
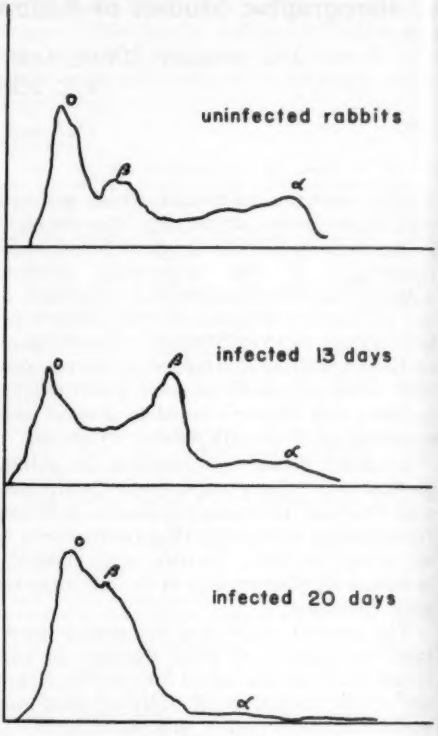


Graph 1—Globulin fraction in relation to control.

ter method gave cleaner peaks, less background, and less variability between duplicates. These results indicate that, as liver damage becomes more severe, the β-lipoprotein component is increased and the α-component becomes less apparent. Rabbits with a 20-day coccidia infection showed somewhat the same lipoprotein picture as human beings with biliary cirrhosis.[5] This may be a specific indication of liver damage or, since the same pattern is obtained following cortisone injection,[2] an indirect effect due to the possibility that severe liver damage interfered with deactivation of endogenous adrenal steroids.

An interesting finding in these experiments was the wide separation between the O-component and the β-lipoprotein fraction occurring at about the thirteenth day. It appears that the α-fraction was increased both in amount and mobility. This lipoprotein change may account for the increased β-globulin occurring at this time. The lipoprotein patterns of the control rabbits and rabbits infected 13 and 20 days are illustrated (graph 2).



Graph 2—Lipoprotein ionographs.

All animals designated as infected were

TABLE 1—Serum Proteins of Rabbits Infected with Eimeria Stiedae

No. days after infection	Gm. per 100 ml. serum				
	Total protein	Albumin	α-globulin	β-globulin	γ-globulin
Control	6.12	3.56	0.98	0.78	0.87
3 days	7.31	3.60	0.95	1.10	1.51
Control	6.88	3.80	1.16	1.02	1.00
6 days	6.89	3.70	1.05	1.10	1.26
Control	6.56	3.55	0.90	1.02	0.96
10 days	6.34	3.29	0.92	0.95	0.88
Control	6.77	4.02	1.00	0.92	0.92
13 days	6.89	3.35	1.09	1.70	1.08
Control	6.30	3.74	0.83	1.11	0.77
17 days	6.69	3.71	0.87	1.25	1.17
Control	6.99	4.00	0.95	1.04	0.91
20 days	7.11	3.52	0.95	1.37	1.08
Control	7.00	4.43	0.97	0.96	0.71
24 days	7.18	3.85	1.08	1.54	0.69
Statistical summary			F value		
Infection effect	1.0	3.75	1.0	13.88*	12.98*

* $P < 0.01$

TABLE 2—Serum Lipoprotein Fractions of Rabbits Infected with Eimeria Stiedae

Days postinfection	Mean Total stainable lipid (%)	
	α	β + O
Control	26.3	73.7
3	19.0	81.0
6	13.6	86.4
10	12.9	87.1
13	11.8	88.2
17	17.6	82.4
20	5.0	95.0
24	17.3	82.7

found, histologically, to have hepatic coccidiosis.

SUMMARY

Protein and lipoprotein fractions of serum were analyzed using ionography (paper electrophoresis). Serum was obtained from rabbits infected with *Eimeria stiedae* at various intervals during the infection.

1) There was a significant increase ($P<0.01$) in both β- and γ-globulin during the course of the infection. The increase in β-globulin was most apparent at the thirteenth day, with an increase also at the third day. The increase in γ-globulin occurred at the third and seventeenth days of infection.

2) The control lipoprotein patterns were similar to those previously reported. By the thirteenth day, the β-lipoprotein had increased in both amount and mobility and the α-fraction was becoming inapparent. At the twentieth day, there was one large peak of O- and β-component with very little α-component.

References

[1] Boguth, W.: Paper-Electrophoretic Serum Examinations of Domestic Animals. Zentralbl. f. Vet.-med., *1*, (1954): 311–329.

[2] Bossak, E. T., Wang, Chun-I., and Adlersberg, D.: Comparative Studies of Lipoproteins in Various Species by Paper Electrophoresis. Proc. Soc. Exptl. Biol. & Med., *87*, (1954): 637–643.

[3] Comer, E. O'B., Swartswelder, J. C., and Jones, C. A.: Electrophoresis Studies of Plasma Proteins in Dogs with Amebic Dysentery. J. Parasitol., *42*, (1956): 25.

[4] Kraut, N.: An Electrophoretic Study of Sera from Rats Artificially Infected with and Immunized Against the Larval Cestode, *Cysticercus Fasciolaris*. J. Parasitol., *42*, (1956): 109–121.

[5] Kunkel, H. G., and Slater, R. J.: Lipoprotein Patterns of Serum Obtained by Zone Electrophoresis. J. Clin. Invest., *31*, (1952): 677–684.

[6] McDonald, H. J.: A New Approach to the Staining of Lipoproteins. In "Paper Electrophoresis," CIBA Foundation Symposium, Little, Brown & Co., Boston (1956): 183–186.

[7] Milch, L. J., Weiner, N., and Robinson, L. G.: Variability Studies of the Flotation Technique of Analytical Ultra-Centrifugation. School of Aviation Medicine, U.S.A.F. Rep. 56–47, 1956.

[8] Spinco Model R, Paper Electrophoresis System, Operating Instructions. Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif., 1954.

[9] Spinco Div.: Lipoprotein Analysis: A Provisional Method, Using the Spinco Model R Paper Electrophoresis System. Spinco Technical Bull. TB6043A, 1956.

[10] Stoloff, I. L., and Abrahams, H.: Filter Paper Electrophoresis. Med. Tech. Bull. 7, (1956): 233–243.

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Studies on the Relationship of a Phospholipase to the Hemolysin of *Leptospira Pomona*

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EVIDENCE that the clinical signs of bacillary hemoglobinuria in cattle (anemia and hemoglobinuria) resemble those of acute, fatal, bovine leptospirosis suggests that the pathogenesis of the two diseases may be similar. The causative agent of the former, *Clostridium hemolyticum*, produces a hemolysin which closely resembles the *alpha*-toxin, a lecithinase, of *Clostridium perfringens welchii*.[8] Recently, it has been reported that a hemolysin is produced by certain species of leptospiras, and that it may be implicated in disease production.[1, 13, 16] It is, therefore, the object of these investigations to point out a possible relationship between the hemolysin produced by pathogenic leptospiras and the *alpha*-toxins (lecithinases) of the clostridia, and also to further characterize this leptospiral hemolysin.

REVIEW OF LITERATURE

MacMillan,[13] Alexander,[1] and Russell[16] demonstrated a leptospiral hemolytic substance present in pathogenic cultures of leptospiras and have designated this factor as a "hemolysin." They suggest that this hemolysin may be implicated in the pathogenesis of leptospirosis. Fizette[6] has suggested that the substrate of this hemolysin may possibly be a phospholipid present at the surface of various cells of the body.

Hemolytic factors have been observed with other microorganisms; in particular, members of the genus Clostridium. MacFarlane[10-12] has observed that the *alpha*-toxin produced by *Cl. perfringens* is capable of splitting the substrate (lecithin), present at the surface of erythrocytes, into a diglyceride and a phosphocholine. *Clostridium hemolyticum*, the causative agent of bacillary hemoglobinuria in cattle, also produces a substance which closely resembles the *alpha*-toxin produced by *Cl. perfringens*.[8]

Pertaining to the *in vitro* activity of lecithinase, time, temperature, and pH may be inhibitory,[3] as may chemical substances such as amino acids[7] and surface active agents.[20] Homologous antiserum has also been noted to inhibit the action of lecithinase.[20] Zamecnik succeeded in protecting mice and dogs against the *alpha*-toxin of *Cl. perfringens* by injection of purified lipids.[21]

Regarding the mechanism whereby hemolytic lecithinases bring about destruction of erythrocytes, the process which apparently accounts for hemolysis is the splitting of lecithin into a diglyceride and a phosphocholine.[9] Whether this occurs is greatly dependent upon the availability and content of lecithin and related phospholipids[19] which make up the complex lipid-protein coaservate present at the surface of erythrocytes.[15]

MATERIALS AND METHODS

The leptospiral hemolysin was prepared as follows:[6] Cultures of *Leptospira pomona* (EHB)* were grown in 16-oz. cotton-stoppered prescription bottles, each containing 400 ml. of Schüffner's medium prepared using a modification of Schubert's technique.[17] The inoculum was 1 ml. of a heavy culture of the organisms. Heavy growth of leptospiras was observed after 16 days. This culture medium, which represents the hemolysin as used in the following studies, was then poured into 4-oz. prescription bottles and stored at −50 C.

Titration for the determination of hemolysin activity was carried out as follows: To each 1.0 ml. of medium titrated, 0.1 ml. of a stock solution (99 mg./ml.) of NaCl was added to make the solution isotonic. The first tube in the titration, prior to the addition of the erythrocyte indicator, contained 1.0 ml. of the nonisotonic culture, 0.1 ml. of the salt stock solution, and 0.9 ml. buffered physiological saline solution (pH 7.3). The contents of the first tube was mixed, and doubling dilutions in 1.0-ml. quantities were made, using buffered saline solution as the diluent. One milliliter of a 1 per cent suspension of packed erythrocytes, which had been previously washed three times with sterile saline solution, was then added to each tube. By this procedure, the dilution of hemolysin was 1:4 in the first tube. Incubation was for two hours at 37 C.

For observing *in vitro* neutralization of hemolysin using lecithin † and other phospholipid-like compounds,** a routine, twofold dilution of hemolysin in 1-ml. amounts was employed using buffered saline solution. To these tubes was added 1 ml. of nonlytic dilutions of the alkaloid substances (table 2). The tubes were then incubated at 37 C. for 40 min., after which 1 ml. of a 1 per cent suspension of packed sheep erythrocytes was

From the Department of Bacteriology, Ohio State University, Columbus.

* Recently isolated from an aborted swine fetus.

† Obtained from the Pfanstiehl Laboratories, Waukegan, Ill.

** Obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, and from the Mathieson Coleman & Bell Co., Norwood, Ohio.

TABLE 1—The Activity of a Leptospiral Hemolysin on the Erythrocytes of Various Animal Species

Hemolysin dilution	Erythrocyte source							
	Chicken	Guinea pig	Mouse	Man	Hamster	Rabbit	Cattle	Sheep
1:4	1*	3	4	2	4	4	3	4
1:8	1	3	4	2	4	3	3	4
1:16	0	2	4	2	3	2	2	4
1:32	....	1	2	1	2	1	2	4
1:64	....	0	0	1	2	1	2	3
1:128	....	....	....	0	1	1	1	2
1:256	....	....	....	....	0	0	1	1
1:512	....	....	....	....	....	....	1	1
1:1,024	....	....	....	....	....	....	0	1
1:2,048	....	....	....	....	....	....	....	0

* Level of hemolysis recorded as 0 to 4 on the basis of visual inspection.

added to each tube. Reincubation was at 37 C. for two hours.

Sheep erythrocyte stroma was prepared after the method described by Beach *et al.*[2] For any adsorption tests, 5 ml. of the packed stroma which had been previously washed three times with sterile saline solution was added to 5 ml. of the hemolysin. The mixture was then centrifuged and the supernatant titrated against sheep erythrocytes.

Animal protection tests were carried out using female golden hamsters,‡ 3 to 5 weeks old. A modification of Zamecnik's method[21] for preparing lecithin emulsions was used as follows: To 1 Gm. of the phospholipid was added 10 ml. of sterile saline solution. The mixture was then heated to 50 C. for two to three minutes, and then allowed to stand for 24 hr. at room temperature. It was then centrifuged at a low rate of speed for five minutes to remove large particles. The emulsified lecithin was then decanted and was ready for use. The nature of the dose administered in these protection tests is given, together with the results (table 3).

RESULTS

Data concerning time and temperature variables were similar to those obtained by Alexander[1] and Russell.[16] The hemolysin is relatively stable upon standing at room temperature for at least 30 hr. Its optimal activity is within the temperature range at 35 to 38 C., whereas it is inactive below 25 C. and above 45 C.

Freshly prepared washed chicken erythrocyte stroma, when mixed with undiluted hemolysin, yielded a white floc. In a like fashion, lecithin plus hemolysin will also yield the same type of floc.

When hemolysin was titrated against erythrocytes of various animal species, in increasing order of activity chicken red blood cells were the most resistant, followed by guinea pig, mouse, human, hamster, rabbit, bovine, and ovine erythrocytes (table 1).

The effect that nonlytic dilutions of lecithin and related compounds have on hemolysin activity *in vitro* is shown (table 2). The hemolysin appears to be most neutralized by cephalin. Sodium lauryl sulfate and saponin, which have been described as excellent inhibitors against the *alpha*-toxin of *Clostridium welchii*,[23] also showed some inhibition of the leptospiral hemolysin.

‡ Obtained from the Lakeview Animal Farm, Lakeview, N. J.

TABLE 2—The Inhibitory Effect of Lecithin, Cephalin, and Surface-Active Agents on Hemolysin Activity

Hemolysin dilution	Substance tested				
	Lecithin (1:1,280)*	Cephalin (1:200)*	Sodium lauryl sulfate (1:3,200)*	Saponin (1:128)*	Hemolysin vs. sheep erythrocytes †
1:4	2**	1	2	2	4
1:8	2	0	2	2	4
1:16	2	....	1	2	4
1:32	1	....	1	1	3
1:64	1	....	1	1	3
1:128	1	....	0	1	3
1:256	0	....	....	0	2
1:512	....	....	....	....	2
1:1,024	....	....	....	....	1
1:2,048	....	....	....	....	0

* Represents the lowest dilution that was not hemolytic. † Control titration. ** Level of hemolysis recorded as 0 to 4 on the basis of visual inspection.

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Lecithin offered some degree of protection (prolongation of life) to hamsters previously inoculated with a pathogenic culture of *L. pomona*. The greatest protection was afforded when 3 to 4 per cent emulsions of the phospholipid were administered intraperitoneally in 0.5-ml. quantities to the animals 15 minutes and one hour after injection of the infective doses (table 3). A lesser degree of protection was offered when the same concentrations of lecithin were administered at 24-hr. intervals, beginning 24 hr. after injection of the organisms.

DISCUSSION

Since optimal activity of the hemolysin is at 37 C., an enzymatic process seems to be implicated. In general, inactivation of enzymes occurs at temperatures above 50 C., with the optimum temperature for activity being about 40 C.

Our results point out the resistance of chicken erythrocytes to the leptospiral hemolysin, in contrast to the susceptibility of sheep erythrocytes. A possible explanation may be that the availability of stroma phospholipid determines the susceptibility of erythrocytes of different animal species to the hemolysin. Supporting this idea, Turner [19] reported that chicken cell surfaces are high in phospholipid content and that this phospholipid is tightly bound; with sheep cell stroma there is a slightly lower content of phospholipids, but the phospholipids are loosely bound to stroma protein. Also, Erickson [5] reported that when stroma is prepared from erythrocytes, all the erythrocyte lipid is contained in the stroma, and that the amount of lipid is related to the size of the cell. Thus, avain erythrocytes are higher in lipid content than are the ovine or bovine cells. However, the erythrocytes of birds contain a tightly bound lipid-protein complex, while those of sheep and cattle are made

TABLE 3—The Protection of Leptospira-Infected Hamsters, Using Lecithin

Animal No.	Description and nature of dose received	Time of death*
1	0.5 cc. 4.0 per cent lecithin	survived
2	0.5 cc. 3.5 per cent lecithin	survived
3	0.5 cc. 3.0 per cent lecithin	survived
4	0.5 cc. 2.0 per cent lecithin	survived
5 & 6	0.3 cc. of a culture of *L. pomona*	90–94 hr.
7 & 8	0.3 cc. of a culture of *L. pomona* followed by two injections of 4.0 per cent lecithin, each of 0.5-cc. quantities. One injection was given 15 min. after injection of organisms; the other 1 hr. after injection of the organisms.	340–348 hr.
9 & 10	0.3 cc. of a culture of *L. pomona* followed by two injections of 3.5 per cent lecithin in 0.5-cc. quantities, both injected in the same manner as animals 7 and 8.	340–348 hr.
11 & 12	0.3 cc. of a culture of *L. pomona* followed by two injections of 3.0 per cent lecithin in 0.5-cc. quantities both injected in the same manner as with animals 7 and 8.	300–312 hr.
13 & 14	0.3 cc. of a culture of *L. pomona* followed by two injections of a 2.0 per cent lecithin emulsion in 0.5-cc. quantities, both injected in the same manner as animals 7 and 8.	235–240 hr.
15 & 16	0.3 cc. of a culture of *L. pomona*. 0.5 cc. of a 4 per cent lecithin emulsion injected starting 24 hr. after injection of the organisms and continued at 24 hr. intervals.	208–214 hr.
17 & 18	0.3 cc. of a culture of *L. pomona*. 0.5 cc. of a 3.5 per cent lecithin emulsion injected in the same manner as with animals 15 and 16.	254–260 hr.
19 & 20	0.3 cc. of a culture of *L. pomona*. 0.5 cc. of a 3.0 per cent emulsion of lecithin injected in the same manner as with animals 15 and 16.	114–120 hr.
21 & 22	0.3 cc. of a culture of *L. pomona*. 0.5 cc. of a 2.0 per cent lecithin emulsion injected in the same manner as with animals 15 and 16.	114–120 hr.
23	0.5 cc. of a 4.0 per cent lecithin emulsion injected at 24 hr. intervals for 12 days.	survived
24	0.5 cc. of a 3.5 per cent lecithin emulsion injected as with animal 23.	survived
25	0.5 cc. of a 3.0 per cent lecithin emulsion injected as with animal 23.	survived
26	0.5 cc. of a 2.0 per cent lecithin emulsion injected as with animal 23.	survived
27	0.5 cc. physiological saline solution	survived
28	No injection	survived

* Period during which both animals died following injection with *L. pomona*.

up of loosely bound lipid-protein complexes. Our results, at this point, seem to indicate that hemolysin activity may be a function of phospholipid availability rather than actual content.

In our investigations, erythrocyte stroma, when mixed with hemolysin, yielded a white floc as does lecithin when it is mixed with hemolysin. This apparent breakdown of a phospholipid, yielding a white floc, has been described as occurring with hemolysins of *Cl. hemolyticum*,[8] and the *alpha*-toxin of *Cl. welchii*.[10,11] In *Cl. welchii*, the hemolysin responsible for the breakdown of the host's erythrocytes has been termed a lecithinase.[11] However, lecithinases are capable of degrading numerous other phospholipids besides lecithin.[4] It seems possible that breakdown of erythrocytes by a leptospiral hemolysin may be due to the action of an enzyme against the phospholipids present on the surface of mammalian erythrocytes.

Lecithin partially inhibited the activity of the hemolysin *in vitro*, as did saponin and sodium lauryl sulfate, compounds known to inhibit the *alpha*-toxin of *Cl. welchii*.[13] Cephalin, the direct analogue of lecithin, almost completely inhibited hemolysin activity. According to Van Haynigen,[20] inhibition of bacterial toxins by phospholipids may be due to a substrate competition effect. From the results obtained using the erythrocytes of various animal species, and from the use of various inhibiting substances, it is entirely possible that a phospholipase is responsible for the hemoglobinuria and anemia that are seen in some acute cases of bovine leptospirosis.

Our results obtained from animal protection tests (table 3) resemble those obtained by Zamecnik [21] in his studies of the *alpha*-toxin of *Cl. welchii*. Protection of animals against phospholipases may be due to the phosphatides dividing the action of the toxin between hydrolysis of the added phosphatides and of the cell phosphatides present in the system, provided a sufficiently high concentration of the former was present. This may explain why 2 per cent emulsions of phosphatides offered no protection, whereas a 4 per cent emulsion did.

Studies are being continued in order to determine a more complete identification of the hemolysin.

SUMMARY

1) The investigations regarding the effect of time and temperature variables on a leptospiral hemolysin indicated that the nature and activity of the substance resembled that of an enzyme.

2) The results obtained when hemolysin acted on the erythrocytes of various animal species suggested a relationship between hemolysin activity and phospholipid availability on the surface of erythrocytes. Brief mention was made regarding the role of phospholipases in acute leptospirosis.

3) The observations that erythrocyte stroma, when mixed with diluted quantities of hemolysin, yielded a white floc and that lecithin treated in a like fashion also yielded a white floc, together with inhibition of hemolysin activity by known lecithinase inhibitors, seemed to indicate a parallel between the activity of the *alpha*-toxins of the clostridia and this leptospiral hemolysin.

4) Concentrations of lecithin, varying from 3 to 4 per cent in 0.5-ml. quantities, prolonged the life of hamsters which had been previously injected intraperitoneally with pathogenic cultures of *Leptospira pomona*. A possible relationship between these observations and those made concerning the *alpha*-toxin of *Clostriduim welchii*, together with a suggested mechanism for this protection, is mentioned.

References

[1] Alexander, A. B., Smith, O. H., Hiatt, C. V., and Gleiser, C. A.: Presence of Hemolysin in Cultures of Pathogenic-Leptospiras. Proc. Soc. Exptl. Biol. & Med., *91*, (1956): 205.

[2] Beach, E. F., Erickson, B. N., Bernstein, S. S., Williams, H. H., and Macy, T. G.: The Amino Acid Composition of Erythrocyte Posthemolytic Residue of Five Mammalian Species. J. Biol. Chem., *108*, (1938): 399.

[3] Collier, H. B.: Factors Affecting the Hemolytic Action of Lysolecithin Upon Rabbit Erythrocytes. J. Gen. Physiol., *35*, (1952): 617.

[4] Dueull, H. J.: The Lipids. Interscience Publishers, New York, N. Y., (1951): 408–439.

[5] Erickson, B. N.: The Lipid Distribution of Posthemolytic Residue of Stroma Erythrocytes. J. Biol. Chem., *122*, (1938): 515.

[6] Fizette, W. B.: Studies on the Pathogenesis of Acute Infections Caused by *Leptospira Pomona* in the Hamster. Ph.D. dissertation, Ohio State University, Columbus, 1957.

[7] Gooder, H., and Gehring, L. B.: Inhibition by Cystine of Lecithinase Production in *Clostridium Perfringens*. Nature, *174*, (1954): 1054.

[8] Jasmin, A.: Enzyme Activity of *Clostridium Hemolyticum*. Am. J. Vet. Res., *8*, (July, 1947): 289–293.

[9] Latta, J.: The Effect of Lipid Solvents on the Surface of Mammalian Erythrocytes. Blood, *7*, (1952): 508.

[10] MacFarlane, M. G.: Hemolysis and the Production of Opalescence in Serum and Lecithinoviteline by the *Alpha*-Toxin of *Clostridium Welchii*. J. Path. & Bact., *52*, (1941): 99.

XUM

[11] MacFarlane, M. G., and Knight, B. J.: The Lecithinase Activity of *Clostridium Welchii* Toxins. Biochem. J., *35*, (1941): 884.

[12] MacFarlane, M. G.: The Enzyme Specificity of *Clostridium Welchii* Lecithinase. Biochem. J., *42*, (1948): 587.

[13] MacMillan, A. E.: A Study of the Methods Used to Demonstrate Leptospiral Antibodies. M.S. thesis, Ohio State University, Columbus, 1953.

[14] Magler, O.: Observations of a Reaction Between the Lethal Toxin of *Clostridium Welchii* (A) and Human Serum. Brit. J. Exptl. Path., *20*, (1939): 473.

[15] Pithica, B. A., and Anderson, P. I.: Role of Stroma Phospholipids and Cholinesterase in Hemolytic Reactions. Colliquium. Biochem. Problemn., Lipidem., *129*, (1953): 129–136.

[16] Russell, C. M.: A Hemolysin Associated with the Leptospira. J. Immunol., 77, (1956): 405.

[17] Schubert, J. H.: A Simplified Procedure for the Preparation of Schuffner's Medium for the Leptospira. Public Health Report, May, 1953.

[18] Smith, L.: The Essential Characteristics of the Species *Clostridium Hemolyticum*. J. Bact., *65*, (1955): 222.

[19] Turner, J. C.: The Absence of Lecithin from Stromata of the Red Cells of Certain Animal Species. J. Exptl. Med., *105*, (1957): 189.

[20] Van Heynigen, W. E.: The Proteinases of *Clostridium Histolyticum*. Biochem. J., *34*, (1940): 1540.

[21] Zamecnik, P. K., Folch, I., and Brewster, L.: Protection of Animals Against *Clostridium Welchii* Toxin by Injection of Purified Lipids. Proc. Soc. Exptl. Biol. & Med., *60*, (1945): 33.

[22] Zamecnik, P. K., and Lipman, J.: Inhibition of *Clostridium Welchii* Toxin and Antitoxin by Lecithin. J. Exptl. Med., *85*, (1947): 395.

[23] Zeller, E. A.: The Enzymes. Academic Press, New York, N. Y., (1951): 986–1013.

Identification and Determination of Kynurenin and 3-Hydroxykynurenin in Bovine Urine

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IN RECENT YEARS, there has been intensive investigation of tryptophan metabolism. A number of tryptophan metabolites have been identified in the urine of different species of animals.[7, 11, 12, 18]

Dunning *et al.*[15] reported that rats fed both tryptophan and 2-acetylaminofluorene developed bladder tumors in high incidence, while those fed 2-acetylaminofluorene alone did not. Tryptophan is a precursor of several aromatic amines[9] chemically similar to those known to produce bladder cancer in man and the dog.[20] Therefore, it was of interest to study the tryptophan metabolites in urine from patients with and without neoplasms of the urinary bladder.[4, 8] Recently, Boyland and Williams[5] reported that 3-hydroxykynurenin and 3-hydroxyanthranilic acid were present in greater than normal amounts in the urine of patients with bladder cancer. Boyland and Watson[3] and Allen *et al.*[1] stated that 3-hydroxyanthranilic acid, 3-hydroxykynurenin, and 2-amino-3-hydroxyacetophenone were carcinogenic when implanted in the bladders of mice.

As far as the authors are aware, there is no report of tryptophan metabolites in cow urine. However, neoplasms of the urinary bladder are common in this species of animal in certain geographic areas.[30-32] Therefore, a study of tryptophan metabolites in urine from cows with and without cancer of the bladder was initiated. This paper reports the identification and determination of kynurenin and 3-hydroxykynurenin in the urine of healthy dairy cows.*

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* The words, kynurenin and 3-hydroxykynurenin, frequently appear with a terminal "e." However, the Editorial Department of the AVMA uses as its authority the Dorland Medical Dictionary, in which the final "e" is omitted.

EXPERIMENTAL PROCEDURE AND RESULTS

Ion-Exchange Chromatography.—The procedure followed in this study was that described by Brown and Price[7] and Brown.[6] The urine samples were collected under toluene from normal dairy cows of the University of Wisconsin dairy farm. One-liter quantities of urine from each of 4 cows were pooled, acidified to 0.1 N with HCl, and the precipitate which developed overnight was removed by centrifugation.

The urine sample was passed through a column of Dowex 50 (H^+), 5.5 cm. in diameter and 11.0 cm. long, followed by 11.0 liters of 0.5 N HCl and then by 50.0 liters of water. Price and Dodge[38] showed that this large volume of water removed certain quinoline compounds from Dowex 50 (H^+). The column then was washed with 2.8 liters of 1.0 N, 2.4 N, and 5.0 N HCl. The last fraction contained kynurenin and 3-hydroxykynurenin.[6, 7] The 5.0 N HCl fraction was vacuum-distilled to dryness at 25 to 32 C. under nitrogen and a dark brown residue was obtained. When this residue was dissolved in water and a small aliquot was mixed with 1 cc. of 0.5 per cent $NaNO_2$ solution in HCl, a strong yellow color developed, which indicated the presence of an *o*-aminophenol.[6]

Paper Chromatographic Investigation.—The 5.0 N column fraction obtained as described was examined by ascending paper chromatography, using Whatman No. 1 or No. 3 MM paper. The solvent systems used were: methanol, butanol, benzene, and water (2:1:1:1), as described by Mason and Berg,[25] containing 1 ml. of glacial acetic acid per 100 ml. of the solvent; 6 per cent sodium acetate containing 1 per cent glacial acetic acid; and the organic phase from a freshly prepared mixture of butanol, acetic acid, and water (4:1:5).[33]

The ratio of the distance of migration of the compound responsible for the color reactions or ultraviolet fluorescence to the distance traveled by the solvent from the point of application of the sample (R_F value) was determined at room tempera-

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ture, and reference standards were run simultaneously on each chromatogram. Seven properties or reactions were used for the routine examination of the chromatograms. These included (1) fluorescence under ultraviolet illumination, (2) ninhydrin reaction, (3) Ehrlich's reaction, (4) Ekman's reaction, (5) Pauly's reaction, (6) reaction with N,2,6-trichloroquinoneimine in ethanol followed by dilute sodium tetraborate, and (7) ammoniacal silver nitrate reaction.[10, 11, 13, 17] Kynurenin gives a positive reaction with Ehrlich's reagent, Ekman's reagent, ninhydrin, and ammoniacal silver nitrate, while 3-hydroxykynurenin gives color reaction with all these reagents. The use of a wide variety of reagents gives more reliable identification of the spots and aids in the detection of contaminating chemicals.

The reactions of spots encountered on paper chromatograms of the 5 N HCl fraction of the Dowex 50 columns are summarized (table 1). Spots C and D, identified as kynurenin and 3-hydroxykynurenin, were eluted from the paper chromatograms with 0.1 N HCl, and the dilute HCl was removed at room temperature over calcium chloride and sodium hydroxide in a vacuum desiccator. These substances were then rechromatographed on paper with the sodium acetate and acetic acid solvent system. The kynurenin and 3-hydroxykynurenin spots showed R_F values and color reactions similar to the authentic substances. The kynurenin and 3-hydroxykynurenin again were eluted from the papers with 0.1 N HCl, taken to dryness as before, and applied to Whatman No. 1 filter paper. The chromatograms were developed in the butanol, acetic acid, and water solvent.

Spectroscopic Investigation.—Kynurenin and 3-hydroxykynurenin were eluted from the chromatograms with 70 per cent methanol. After evaporation of the methanol, their spectra were determined between 230 and 440 mμ in 0.1 M phosphate buffers at pH's of 2.0, 7.4, and 12.0, using a Beckman model DU spectrophotometer. The kynurenin fraction in these buffers showed spectra almost identical to those of authentic kynurenin.

Although 3-hydroxykynurenin from cow urine seemed pure on paper chromatograms developed in three solvent systems, some substance interfered with the spectral readings at the longer wave lengths.

Colorimetric Investigation.—Aliquots of kynurenin and of 3-hydroxykynurenin eluted from paper chromatograms with 0.1 N HCl were used for colorimetric investigations. The method followed in this study was that described by Brown and Price[7] and Brown,[6] with the omission of the step which involved treatment of the fraction with 9.0 N NaOH. Standard tubes (10 μg. of kynurenin and 20 μg. of 3-hydroxykynurenin/tube) were prepared under the same conditions. The optical densities of these derivatives of kynurenin and 3-hydroxykynurenin were measured in the Beckman spectrophotometer.

Kynurenin isolated from cow urine and the authentic compound showed maximum absorption at 550 mμ. The maximum readings with both the isolated and the authentic 3-hydroxykynurenin were obtained at 410 mμ.[6, 7]

Enzyme Study.—Kynurenin transaminase, an enzyme from *Neurospora crassa* or

TABLE 1—Summary of Color Reactions Obtained after Ascending Chromatography *

Spots	Mean R_F	Color reactions							
		Fluorescence under ultraviolet light	Ninhydrin	Ehrlich's reagent	Ekman's reagent	Pauly's reagent	TCQ † reagent	Ammonical silver nitrate	Identity
A	0.34	Yellow-green	Purple	Orange-pink with delay	.....	Pink-brown	.....	.....	.....
B	0.41	Blue	.....	Orange-pink with delay	.....	Pink-brown	Light gray	.....	.....
C	0.45	Green	Brown-purple	Orange-pink with delay	Yellow, later brown	Red-brown	Dark gray	Brown-black	3-Hydroxy-kynurenin
D	0.52	Strong pale blue	Purple	Orange immediately	Purple	.....	.....	Red-brown with delay	L-Kynurenin
E	0.68	Blue	.....	.....	.....	.....	.....	.....	.....
F	0.81	Yellow-green	.....	.....	.....	.....	.....	.....	.....

* On Whatman No. 1 paper in Mason and Berg's solvent containing 1 per cent acetic acid (time: 16 hr.).
† N,2,6-trichloroquinoneimine in ethanol, followed by sodium borate buffer, pH 9.0.

from rat liver and kidney homogenates, has been shown to transaminate kynurenin and 3-hydroxykynurenin to kynurenic acid and xanthurenic acid, respectively. A variety of *alpha*-ketoacids act as amino group acceptors; pyridoxal phosphate is essential for the enzyme action.[21,24-26,39]

Kynurenin and 3-hydroxykynurenin isolated from cow urine were subjected to the kynurenin transaminase system of washed rat liver mitochondria, as described by De Castro *et al.*[14] The incubation medium consisted of concentrated eluates of kynurenin and 3-hydroxykynurenin from paper chromatograms in water, 0.5 ml.; pyriodoxal phosphate, 80.0 μg.; *alpha*-ketoglutarate, 60.0 μM; 0.05 M potassium phosphate buffer, pH 7.4; mitochondria from 1 Gm. of rat liver, 1.0 ml. At the same time, 5.0 μM of known kynurenin and about 4.5 μM of 3-hydroxykynurenin also were incubated under the same conditions. A blank which contained all materials but kynurenin and 3-hydroxykynurenin served as a control. All flasks were made to a final volume of 2.0 ml. with water. Incubations were carried out in stoppered 25.0-ml. Erlenmeyer flasks which were shaken in a water bath at 37 C. After 180 minutes' incubation, the incubation mixtures were deproteinized by dipping the flasks into boiling water for one minute. The supernatant fluids were kept frozen until used. Paper chromatograms prepared with these supernatant fluids were developed in Mason and Berg's[26] solvent containing 1.0 ml. of 15 N NH_4OH per 100.0 ml. of the solvent.[38] The chromatograms were examined under ultraviolet illumination and sprayed with Pauly's reagent and 1.7 per cent $FeNH_4(SO_4)_2 \cdot 12\ H_2O$, followed by 5.0 per cent $NaHCO_3$.[12,38] Known kynurenic acid and xanthurenic acid were chromatographed simultaneously.

The results are given (table 2). The kynurenin transaminase converted the kynurenin and 3-hydroxykynurenin isolated from cow urine into products which were indistinguishable from kynurenic acid and xanthurenic acid, respectively. Similar results were obtained with the authentic substrates. It appears that the results obtained from paper chromatography and the spectroscopic, colorimetric, and enzyme studies adequately establish the fact that dairy cows on normal rations excrete kynurenin and 3-hydroxykynurenin in the urine.

Determination of Kynurenin and 3-Hydroxykynurenin.—After it was established that cows excrete kynurenin and hydroxykynurenin, the amount of these tryptophan metabolites excreted in the urine was estimated.

The methods used for the determination of kynurenin and 3-hydroxykynurenin in cow urine were essentially those described previously.[6,7] Duplicate 10-ml. aliquots of urine were placed in 50-ml. graduated centrifuge tubes, acidified by the addition of 1 ml. of 4.0 N HCl, and diluted to 40 ml. with water. To one of the tubes, 200 μg. of kynurenin sulfate and 400 μg. of 3-hydroxykynurenin were added for the determination of the recoveries. The diluted samples then were applied to 10-cm. columns of Dowex 50 (H^+) (0.9 cm., inside diameter) and passed through at a flow rate of 20 to 25 drops per minute. The samples were washed through the columns with two 20-ml. portions of 0.1 N HCl. The columns then were washed with 80 ml. of 0.5, 1.0, 2.4, and 5.0 N HCl. The 5.0 N HCl fraction contained the kynurenin and 3-hydroxykynurenin which were measured as described previously.[6,7]

Since it was not practical to collect 24-hour urine samples from cows, the kynu-

TABLE 2—Results of Paper Chromatography of Kynurenic Acid and Xanthurenic Acid Formed from L-Kynurenin and 3-Hydroxy-L-Kynurenin Isolated from Cow Urine and from Authentic Compounds after Incubation with Rat Liver Kynurenin Transaminase

	Substrates incubated					
	Isolated		Authentic		Standard	
Reactions	Hkyn	Kyn	Hkyn	Kyn	XA	KA
R_F in Mason and Berg's ammonia solvent	0.29	0.62	0.29	0.62	0.32	0.62
Ultraviolet fluorescence	Gray-blue	Yellow-green	Gray-blue	Yellow-green	Gray-blue	Yellow-gre n
Color with Pauly's reagent	Red	.....	Red	.....	Red	.....
Color with $FeNH_4(SO_4)_2$	Green	.....	Green	.....	Green	.....

Hkyn = 3-hydroxy-L-kynurenin; Kyn = L-kynurenin; XA = xanthurenic acid; KA = kynurenic acid.

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renin and 3-hydroxykynurenin content of the urine samples was expressed as milligrams per gram of creatinine. The 24-hour urine volume of dairy cows ranges from about 9 to 23 liters,[16] with a creatinine content of about 1.1 Gm. (0.4 to 1.92 Gm.) per liter.[27] The creatinine was determined as described by Peters.[34]

The excretion of kynurenin, 3-hydroxykynurenin, and creatinine by 4 dairy cows has been recorded (table 3). The kynurenin excretion ranged from 7.5 to 19.5 mg. per gram of creatinine, and the excretion of 3-hydroxykynurenin ranged from 6.9 to 29.4 mg. per gram of creatinine.

After the quantitative determination of the kynurenin and 3-hydroxykynurenin content of the 5 N HCl fraction from the Dowex 50 columns, 70 ml. of the remaining fraction was evaporated to dryness *in vacuo* over calcium chloride and sodium hydroxide. The residue was dissolved in 50 per cent ethanol and applied as a band on Whatman No. 1 filter paper. After development with the solvent system of Mason and Berg [25] containing acetic acid, the chromatograms were examined by the procedures summarized (table 1). Kynurenin and 3-hydroxykynurenin were detected readily, but there were no bands to indicate other aromatic amines or aminophenols.

The results of these experiments were regarded as satisfactory evidence for the validity of the ion-exchange chromatographic method of quantitative analysis.

DISCUSSION

Previous studies from these laboratories have shown that kynurenin and kynurenic acid were significant urinary metabolites of tryptophan in the dog, the rat, and man.[7] However, cats excreted little if any kynurenin in the urine.[7] It has been shown that human beings given isoniazid and rats on a low pyridoxine ration excreted 3-hydroxykynurenin as the chief metabolite after the administration of tryptophan.[6,11] The 3-hydroxykynurenin has been shown to be excreted in large amounts by patients with severe tuberculosis,[23] by a large proportion of patients with fevers of varying cause,[13] by patients with leukemia,[28,29] with diabetes,[22] and with scleroderma (acrosclerosis).[37] Human beings with no known disease, as well as tuberculous patients given the pyridoxine antagonists, isoniazid, or deoxypyridoxine, excreted more 3-hydroxykynurenin in the urine than kynurenin.[35,36] The quantitative studies on patients with scleroderma, however, revealed that kynurenin was excreted in larger quantities than 3-hydroxykynurenin after the ingestion of supplements of L-tryptophan.[37]

The occurrence of kynurenin and 3-hydroxykynurenin in the urine of the cow has not been demonstrated previously. The excretion of kynurenin and 3-hydroxykynurenin by the cow apparently is somewhat greater than that of man, in terms of the creatinine content in the urine. Thus, the daily output of cow urine ranges from about 9 to 23 liters,[16] with creatinine values of about 1.1 Gm. per liter,[27] as compared with 0.172 to 0.975 Gm. per liter (average 0.602) found in our study. The daily excretion of creatinine by normal adult human beings ranged between 1.0 and 1.8 Gm.[19] The range of values of excretion of kynurenin and 3-hydroxykynurenin for cows (expressed as mg./Gm. of creatinine) was 7.5 to 19.5 and 6.9 to 29.4, respectively. Human beings excreted 2.0 to 3.0 mg. of kynurenin and 1.0 to 6.0 mg. of 3-hydroxykynurenin daily.[5-7] Thus, on the basis of the total daily excre-

TABLE 3—Excretion of Creatinine, Kynurenin, and 3-Hydroxykynurenin in Urine of Dairy Cows

Cow No. and breed	Creatinine (Gm./liter)	Kynurenin		3-Hydroxykynurenin	
		Milligrams per gram creatinine*	Percentage recovery from column	Milligrams per gram creatinine*	Percentage recovery from column
77 Guernsey	0.957	7.5	80	9.2	118
130 Guernsey	0.172	19.5	88	21.5	120
111 Guernsey	0.952	9.7	86	6.9	111
600 Holstein-Friesian	0.327	9.8	89	29.4	108
Average	0.602	11.6	86	16.8	114

* The values were corrected for column recoveries of standards added to the urine.

tion or expressed per gram of creatinine, the dairy cows excreted more kynurenin and 3-hydroxykynurenin than human beings.

The high levels of kynurenin and 3-hydroxykynurenin found in bovine urine were of more interest in the light of the high incidence of bladder cancer in this species [30-32] in certain districts. Previous studies from these laboratories emphasized that the dog, the rat, and man excreted relatively large amounts of kynurenin and 3-hydroxykynurenin and that all these species develop spontaneous bladder cancer, while the cat, which rarely if ever develops bladder cancer, was found to excrete quantities of these compounds which were below detectable levels.[7] The correlations, together with the apparent relationship of tryptophan metabolism to the induction of bladder cancer in rats with 2-acetylaminofluorene [15] and the relatively large excretion of kynurenin, kynurenic acid, acetylkynurenin,[8] anthranilic acid, 3-hydroxyanthranilic acid, and 3-hydroxykynurenin [5] after the ingestion of tryptophan by patients with bladder cancer, suggest the possibility that some of these metabolites of tryptophan may be of causal significance in mammalian bladder cancer. The recent report that bladder tumors were obtained in mice when 3-hydroxykynurenin, 3-hydroxyanthranilic acid, and 2-amino-3-hydroxyacetophenone were implanted in the bladders of mice provides additional support for this hypothesis.[1,3] The extremely low level of tryptophan metabolites found in cat urine [7] might partly explain the apparent rarity of urinary bladder cancer in this species.[2]

SUMMARY

1) The occurrence of L-kynurenin and 3-hydroxy-L-kynurenin has been demonstrated for the first time in the urine of dairy cows.

2) Dairy cows on normal rations excreted 7.5 to 19.5 mg. of L-kynurenin and 6.9 to 29.4 mg. of 3-hydroxykynurenin per gram of creatinine.

3) The possible relationship between bladder cancer and the urinary metabolites of tryptophan was discussed.

References

[1] Allen, M. J., Boyland, E., Dukes, C. E., Horning, E. S., and Watson, J. G.: Cancer of the Urinary Bladder Induced in Mice with Metabolites of Aromatic Amines and Tryptophan. Brit. J. Cancer, *11*, (1957): 212–228.

[2] Bloom, F.: Pathology of the Dog and Cat. American Veterinary Publications, Inc., Evanston, Ill. (1954): 184.

[3] Boyland, E., and Watson, G.: 3-Hydroxyanthranilic Acid, a Carcinogen Produced by Endogenous Metabolism. Nature, *177*, (1956): 837–838.

[4] Boyland, E., and Williams, D. C.: The Estimation of Tryptophan Metabolites in the Urine of Patients with Cancer of the Bladder. Biochem. J., *60*, (1955): V.

[5] Boyland, E., and Williams, D. C.: The Metabolism of Tryptophan. II. The Metabolism of Tryptophan in Patients Suffering from Cancer of the Bladder. Biochem. J., *64*, (1956): 578–582.

[6] Brown, R. R.: The Isolation and Determination of Urinary Hydroxykynurenine. J. Biol. Chem., *227*, (1957): 649–652.

[7] Brown, R. R., and Price, J. M.: Quantitative Studies on Metabolites of Tryptophan in the Urine of the Dog, Cat, Rat, and Man. J. Biol. Chem., *219*, (1956): 985–997.

[8] Brown, R. R., Price, J. M., and Wear, J. B.: The Metabolism of Tryptophan in Bladder Tumor Patients. Proc. Am. A. Cancer Res., *2*, (1955): 7.

[9] Dalgliesh, C. E.: Biological Degradation of Tryptophan. Quart. Rev. (London), *5*, (1951): 227–244.

[10] Dalgliesh, C. E.: Isolation and Examination of Urinary Metabolites Containing an Aromatic System. J. Clin. Path., *8*, (1955): 73–78.

[11] Dalgliesh, C. E.: The Relation Between Pyridoxin and Tryptophan Metabolism Studied in the Rat. Biochem. J., *52*, (1952): 3–14.

[12] Dalgliesh, C. E., Knox, W. E., and Neuberger, A.: Intermediary Metabolism of Tryptophan. Nature, *168*, (1951): 20–22.

[13] Dalgliesh, C. E., and Tekman, S.: The Excretions of Kynurenine and 3-Hydroxykynurenine. Biochem. J., *56*, (1954): 458–463.

[14] De Castro, F. T., Brown, R. R., and Price, J. M.: The Intermediary Metabolism of Tryptophan by Cat and Rat Tissue Preparations. J. Biol. Chem., *228*, (1957): 777–784.

[15] Dunning, W. F., Curtis, M. R., and Maun, M. E.: The Effect of Added Dietary Tryptophane on Occurrence of 2-Acetylaminofluorene-Induced Liver and Bladder Cancer in Rats. Cancer Res., *10*, (1950): 454–459.

[16] Fuller, J. M.: Some Physical and Physiological Activities of Dairy Cows Under Conditions of Modern Herd Management. New Hampshire Agric. Exper. Sta. Tech. Bull. 35, (1928): 1–30.

[17] Gibbs, H. D.: Phenol Tests. III. The Indophenol Test. J. Biol. Chem., *72*, (1927): 649–664.

[18] Gordon, W. G., Kaufman, R. E., and Jackson, R. W.: The Excretion of Kynurenic Acid by the Mammalian Organism. A Method for the Identification of Small Amounts of Kynurenic Acid. J. Biol. Chem., *113*, (1936): 125–134.

[19] Hobson, W.: Urinary Output of Creatine and Creatinine Associated with Physical Exercise and Its Relationship to Carbohydrate Metabolism. Biochem. J., *33*, (1939): 1425–1431.

[20] Hueper, W. C.: Recent Developments in Environmental Cancer. Arch. Path., *58*, (1954): 475–523.

[21] Jakoby, W. B., and Bonner, D. M.: Kynurenine Transaminase from Neurospora. J. Biol. Chem., *221*, (1956): 689–695.

[22] Kotake, Y., Jr., and Tani, S.: Studies on Xanthurenic Acid. III. Xanthurenic Acid in the Urine of Diabetic Patients. J. Biochem. (Japan), *40*, (1953): 295–298.

[23] Makino, K., Satoh, K., Fujiki, T., and Kawaguchi, K.: Relation of 3-Hydroxykynurenine to the Ehrlich Diazo Reaction of Urine in Severe Tuberculosis. Nature, *170*, (1952): 977–978.

[24] Mason, M.: The Kynurenine Transaminase of Rat Kidney. J. Biol. Chem., *211*, (1954): 839–844.

[25] Mason, M., and Berg, C. P.: A Chromatographic Method for the Detection of Tryptophan Metabolites. J. Biol. Chem., *188*, (1951): 783–788.

[26] Mason, M., and Berg, C. P.: The Metabolism of D- and L-Tryptophan and D- and L-Kynurenine by Liver and Kidney Preparations. J. Biol. Chem., *195*, (1952): 515–524.

[27] Münzer, H.: Kolorimetrische Kreatinin-und Indikanbestimmungen im Harn der Haustiere nach Autenrieth und Königsberger. Pflüger's Arch. ges. Physiol., *158*, (1914): 41–83.

XUM

[28] Musajo, L.: Recherches sur les métabolites du tryptophane et premières applications à la physiopathologie humaine. Bull. Soc. Chim. biol. (Paris), *35*, (1953): 711–719.

[29] Musajo, L., Benassi, C. A., and Parpajola, A.: Isolation of Kynurenine and 3-Hydroxykynurenine from Human Pathological Urine. Nature, *175*, (1955): 855–856.

[30] Pamukcu, A. M.: Investigations on the Pathology of Enzootic Bovine Haematuria in Turkey. Zentralbl. f. Vet.-Med., *2*, (1955): 409–429.

[31] Pamukcu, A. M.: Pathology of Bovine Haematuria. Rapports of VII^me Cong. Int. Path. Comp., Lausanne, *2*, (1955): 156–157.

[32] Pamukcu, A. M.. Tumors of the Urinary Bladder in Cattle and Water Buffalo Affected with Enzootic Bovine Hematuria. Zentralbl. f. Vet.-Med., *4*, (1957): 185–197.

[33] Partridge, S. M.: Application of Paper Partition Chromatogram to the Qualitative Analysis of Reducing Sugars. Nature, *158*, (1946): 270–271.

[34] Peters, J. H.: The Determination of Creatinine and Creatine in Blood and Urine with the Photoelectric Colorimeter. J. Biol. Chem., *146*, (1942): 179–186.

[35] Price, J. M., Brown, R. R., and Larson, F. C.: Quantitative Studies on Human Urinary Metabolites of Tryptophan as Affected by Isoniazid and Deoxypyridoxine. J. Clin. Invest., *36*, (1957): 1600–1607.

[36] Price, J. M., Brown, R. R., and Larson, F. C.: Quantitative Studies on the Metabolism of Tryptophan in Patients Receiving Isoniazid Therapy. Fed. Proc., *15*, (1956): 330.

[37] Price, J. M., Brown, R. R., Rukavina, J. G., Mendelson, C., and Johnson, S. A. M.: Scleroderma (Acrosclerosis). II. Tryptophan Metabolism Before and During Treatment by Chelation (EDTA). J. Invest. Dermatol., *29*, (1957): 289–298.

[38] Price, J. M., and Dodge, L. W.: Occurrence of the 8-Methyl Ether of Xanthurenic Acid in Normal Human Urine. J. Biol. Chem., *223*, (1956): 699–704.

[39] Wiss, O.: Die Bedeutung des Pyridoxal-5-Phosphates für den Kynurenin-und 3-Oxykynurenin-Abbau. Ztschr. f. Naturforsch., *7B*, (1952): 133–136.

Observations on the Penile Development of the Angora Goat

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VARIATIONS in the development of the penis and its relation to the prepuce have been observed during the course of routine postmortem examinations of adult male goats. Nonseparation of the surface epithelium of the glans penis from the epithelium of the surrounding prepuce and an underdevelopment of the penis are found in most castrated males.

Similar variations have been observed by others in bovine penile development. Getty[2] states that the penis is not free in the prepuce in the young bovine male and that the separation, allowing for protrusion of the penis, occurs at or near the age of puberty. He further states that no detailed studies have been made on the effect of castration when performed before puberty, and of its relationship to the maintenance of the fusion of these layers in later life. Benbrook[1] has observed a lack of separation of the two surfaces in an immature male deer. Marsh and Safford[3] report changes in the diameter of the urethral lumen of males calves as affected by their age at the time of castration.

MATERIALS AND METHODS

This report deals with general observations on the development of the goat penis, as seen during the postmortem examination of 400 white Angora goats. Detailed measurements of the penis in 24 goats are included. This series comprises 8 normal males, 13 castrated males, and 3 males with one testis retained intra-abdominally and the other surgically removed. The detailed measurements are selected from random necropsies and are believed to be representative. The penises from several goats were fixed in formalin, sectioned transversely, and then examined microscopically to determine the extent of the stratified squamous epithelium. Single sections from the fused region of the penises of numerous other goats were also examined.

The animals used in our laboratory were adult Angora goats, most of which are believed to have been castrated at an early age.[5] They were used for the production of mohair and we received them after they were mature and no longer satisfactory for the production of a quality product. The age of the animals was judged by the stage of dentition and, with few exceptions, all had a full set of permanent incisors, indicating that their age was 4½ years or more. We assumed that most of these animals were castrated before puberty, although the penile development suggested that a few animals may have been castrated near or after the time they achieved sexual maturity.

OBSERVATIONS AND DISCUSSION

A few of our experimental animals were normal adult males. The structure of the penis in these animals resembled that described for the ram by Sisson and Grossman.[4] Approximately 4.0 cm. of the anterior tip of the penis was free of the prepuce and the galea glandis or tip was 1.5 to 2.0 cm. long. The urethral process left the body of the penis 1.0 cm. from the tip, extending over the surface of the galea glandis to project 1.0 to 1.5 cm. beyond the tip of the penis. The urethral process itself was 2.0 to 2.5 cm. long. The diameter of the penis at the level of the preputial reflection was 1.0 to 1.4 cm., which was close to the maximum diameter of the penis throughout its length from tip to sigmoid flexure. The length of the preputial cavity varied from 9.0 to 14.0 cm.

In a few of the castrated males, there was a similar separation of the prepuce and penis; the penis was 0.8 to 1.0 cm. in diameter, indicating that these animals may have been castrated at a time closer to sexual maturity than the average goat. The majority of the castrated males had small penises, little or none of which was free in the preputial cavity. The usual castrated goat had only 1.0 to 2.0 mm. of the galea glandis free, as compared to 3.0 to 4.0 cm. of free penis in uncastrated animals. The separation often extended for 1.0 to 2.0 mm. farther posterior on the ventral surface of the penis than on the dorsum. In some cases, the entire urethral process was also fused to the preputial surface. Separation of the two structures was difficult, giving the impression that the union might be connective tissue in nature.

It might first be suspected that this lack of separation of the two epithelial surfaces

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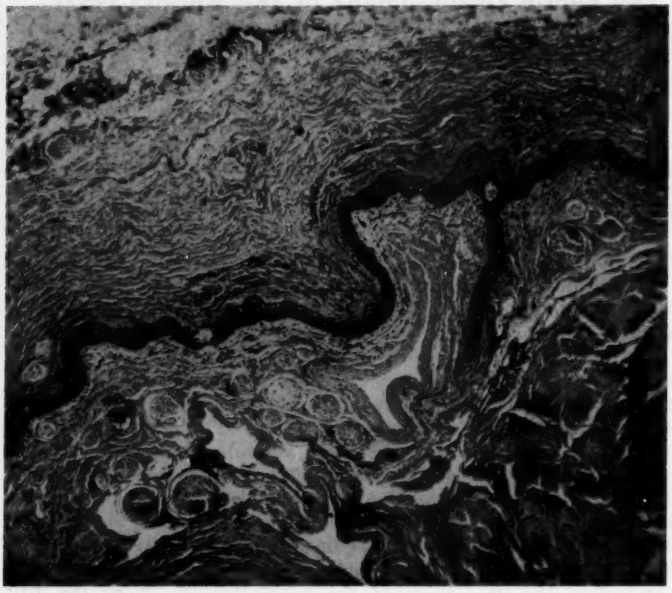


Fig. 1—Part of the ring of epithelium encircling the penis, 6 mm. posterior to the tip of the glans (animal G1340). Penile tissue is below the epithelium and preputial tissue is above. H & E stain; x 50.

was the result of an inflammatory reaction with subsequent formation of connective tissue adhesions; however, microscopic examination revealed no evidence of acute or chronic inflammation and no connective tissue proliferation. There was a solid zone or ring of epithelium encircling the penis which appeared capable of separation into two opposing layers of stratified squamous epithelium (fig. 1). Basal cells were found at the outer and inner borders of the ring of epithelium (fig. 2, 3).

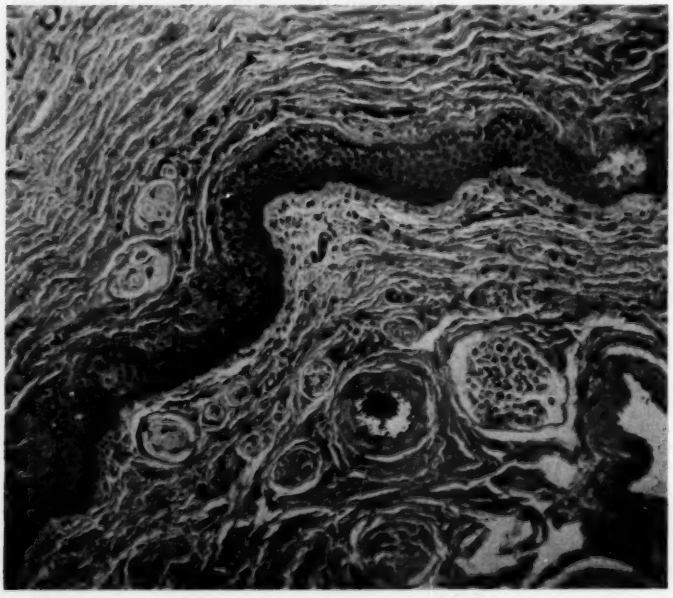


Fig. 2—A higher magnification of the band of epithelium seen in figure 1. H & E stain; x 144.

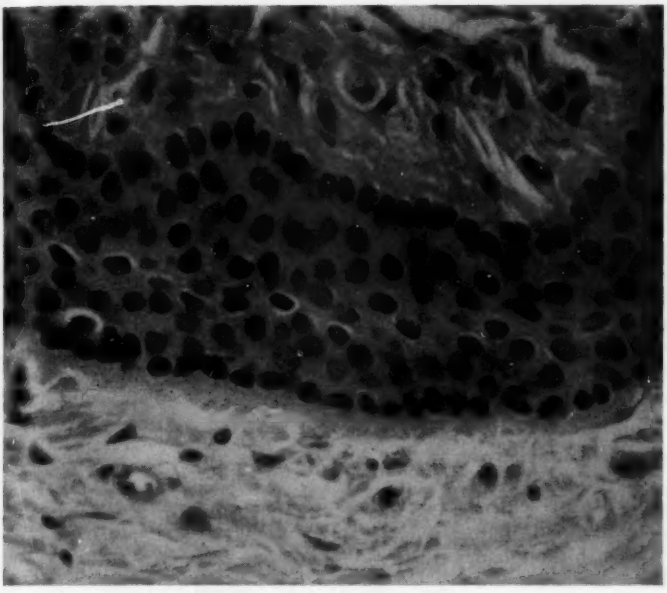


Fig. 3—A higher magnification of the epithelium shown in figure 1, taken at another point along the band encircling the penis. Notice the two opposing layers of basal cells. H & E stain; x 650.

The cells were flatter and more compressed in the center of the band, just as cells near the surface of typical stratified squamous epithelium are flattened. In these cases, there was no separation of the two potential layers of stratified squamous epithelium, and thus there were no exposed surfaces covered by completely flattened or cornified cells. The central part of the band was a solid mass of slightly flattened cells,

TABLE 1—Measurements (in cm.) of the Penises of Goats

Goat No.	Sex	Length of penis, tip to first curve of sigmoid flexure	Length of free penis	Diameter of penis	Length of preputial cavity
G1060	Castrated male	.....	0.0	....	.....
G1078	Castrated male	.....	0.0	....	.....
G1105	Castrated male	.....	0.0	0.6	.....
G1108	Castrated male	.....	0.0	0.5	.....
G1116	Castrated male	.....	4.0	....	.....
G1222	Castrated male	17.0	3.0	0.8	11.0
G1228	Castrated male	16.0	0.3	0.6	8.0
G1231	Castrated male	20.0	4.0	1.0	10.0
G1234	Castrated male	18.0	0.6	0.6	8.0
G1244	Castrated male	18.0	0.6	0.6	8.5
G1266	Castrated male	16.0	0.0	0.7	.....
G1330	Castrated male	21.0	3.0	0.8	13.5
G1349	Castrated male	15.5	0.0	0.6	7.5
range		15.5–21.0	0.0–4.0	0.5–1.0	7.5–13.5
G1329	Male	21.0	3.5	1.2	13.0
G1240	Male	19.0	4.0	1.2	13.5
G1250	Male	19.3	3.7	1.1	9.7
G1334	Male	19.0	4.0	1.3	13.7
G1353	Male	17.0	3.5	1.1	10.0
G1355	Male	18.0	3.0	1.2	9.0
G1375	Male	21.0	4.0	1.2	12.0
G1376	Male	19.0	4.0	1.1	9.0
range		17.0–21.0	3.0–4.0	1.1–1.3	9.0–13.7
G1242	Male, cryptorchid	20.0	3.0	0.8	10.0
G1327	Male, cryptorchid	21.0	4.0	1.2	13.0
G1221	Male, cryptorchid	21.0	4.0	0.8	10.0
range		20.0–21.0	3.0–4.0	0.8–1.2	10.0–13.0

XUM

with no hint of cornification or separation.

In areas near where the two layers did separate, a few tiny slit-like spaces existed in the epithelium. Cells lining the slits were flatter and some of them were cornified. The larger spaces, located nearer to the reflection of the prepuce, were filled with sloughed cornified squamous cells. The ring of epithelium extended for 3 to 4 cm. posterior to the tip of the penis in all cases without regard for the extent of the separation, and the anterior 3 to 4 cm. of the penis was always surrounded by a layer of epithelium, whether free or fused. Posterior to the limits of the epithelium, the penis was surrounded by loose connective tissue of the subcutis.

Measurements on the extent of separation of the penis from the prepuce and the development of the penis are presented (table 1). Most of the castrated goats had only a short tip of free penis, although a few were no different from normal males in this respect. These measurements would be more significant if dates of castration were available.

The penile development of 3 goats with intra-abdominal testes was the same as that of normal males.

SUMMARY

In the course of 400 routine necropsies, it was observed that there was a failure of separation of the epithelial surfaces of the penis and prepuce in the majority of castrated goats, while the penis was free for approximately 4 cm. in normal, uncastrated males.

Upon microscopic examination, it was found that the two layers of stratified squamous epithelium which cover the penis and line the preputial cavity of the castrated male were not separated but formed a solid band of epithelium with two opposing basal layers.

References

[1] Benbrook, S. C., U.S. Medical Research Laboratory, Fort Knox, Ky.: Personal communication, 1957.

[2] Getty, R., Iowa State College, Ames: Personal communication, 1957.

[3] Marsh, H., and Safford, J. W.: Effect of Deferred Castration on Urethral Development in Calves. J.A.V.M.A., *130*, (April 15, 1957): 342–344.

[4] Sisson, S., and Grossman, J. D.: The Anatomy of the Domestic Animals. 4th ed. W. B. Saunders Co., Philadelphia, (1953): 601.

[5] U.S. Department of Agriculture: The Angora Goat. U.S. Government Printing Office, Washington, D.C., Farmers' Bull. 1203, 1946.

Scientific Notes

Microcard Reprints of Research Journal

Microcard reprints of volumes 1 to 17 of the *American Journal of Veterinary Research* are currently available from J. S. Canner and Company, Inc., Library Booksellers, 618 Parker St., Boston 20, Mass. These volumes encompass the years 1940–1956, and sell for $67.50

Virtually any of the various machines designed for microcard print will serve for reading. However, the microcard reader which has been specifically designed for reading microcards is recommended. This particular model ranges from pocket size to the conventional one that rests on a table but is portable enough to be easily carried.

Further information regarding these readers may be obtained from J. S. Canner and Company.

Charter Meeting of Laboratory Animal Breeders Association

The charter meeting of the Laboratory Animal Breeders Association was held in Chicago on Dec. 2, 1958. The membership, now 56, includes most of the large organizations in the country and many producers who operate on a smaller scale.

In the next few years, the L.A.B.A. hopes it will be able to sponsor and participate in a program for accrediting the facilities of laboratory animal breeders.

Since there is a need for expansion of knowledge in the field of laboratory animal husbandry, a Committee on Research has been established to consider problems dealing with the husbandry phase of the industry.

To establish liaison with those in other phases of the industry, primarily the users, an editorial and publicity committee was formed to acquaint other branches of the industry with the organization and its activities. The Advisory Committee consists of men who are nationally known and whose principal interests are in the field of biological investigation. These persons are not members of L.A.B.A. but function as a scientific liaison with the organization.

Guest speakers at the first meeting were Dr. L. R. Christensen, New York University; Mr. Berton F. Hill, Institute of Laboratory Animal Resources; and Dr. W. T. S. Thorp, University of Minnesota.

Meetings • • •

• • • International Veterinary Congress. Sixteenth session. Madrid, Spain, May 21–27, 1959. Prof. Pedro Carda A., general secretary, Calle Villanueva 11, Madrid.

U. S. COMMITTEE: Dr. W. A. Hagan, chairman, New York State Veterinary College, Ithaca, N. Y.; Dr. J. G. Hardenbergh, secretary, 600 S. Michigan Ave., Chicago 5, Ill.

• • • Third World Congress on Fertility and Sterility, Amsterdam, Holland, June 7–13, 1959. Dr. L. I. Swabb, Sint Agnietenstraat 4, Amsterdam, Holland, honorary secretary.

• • • Ninety-Sixth Annual Meeting, American Veterinary Medical Association, and Third Pan American Congress of Veterinary Medicine, Joint meeting. Kansas City, Mo., Aug. 23–27, 1959. H. E. Kingman, Jr., executive-secretary, AVMA, 600 S. Michigan Ave., Chicago 5, Ill. B. D. Blood, secretary-general. Directing Council, Pan American Congress of Veterinary Medicine, P. O. Box 99, Azul, F.C.N.G.R., Argentina, S.A.

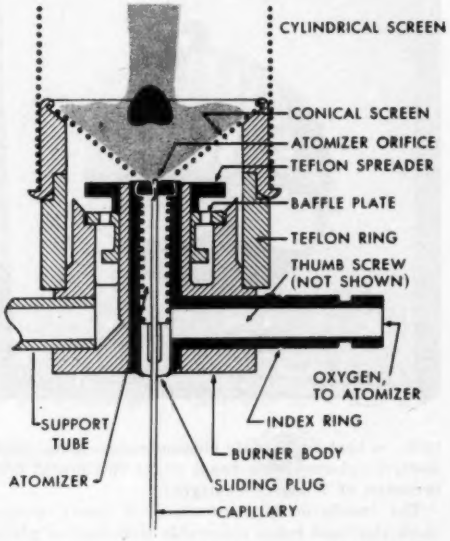
Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

Flame Photometer

Diverse materials can be analyzed for precise determinations of sodium, potassium, and calcium with the Coleman Model 21 Flame Photometer, manufactured by Coleman Instruments, Inc.

An atomizer-burner combines the fuel gas with



XUM

oxygen to form a stable, high-temperature flame; liquid samples are atomized into the base of this flame. The light energy radiated by the flame is measured by a direct-coupled, prealigned phototube photometer with a 115-volt AC line-operated amplifier.

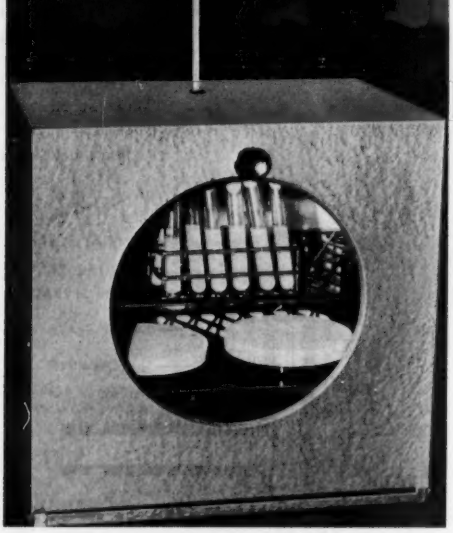
Optical filters isolate the radiation of the element under analysis. Filters offered with the instrument are adjusted for measurement of sodium, potassium, and calcium in blood plasma, serum, urine, and other solutions with similar concentrations of the element. Blank holders are available for filters adjusted for other solutions.

Sample readings are obtained from the galvanometer scale of the external reading instrument, which is connected through a plug-in cable.

Bacteriological Incubator

A new incubator for routine bacteriological procedures in the veterinary practice is available from Norden Laboratories.

The incubator is 12½ inches high, 12¼ inches wide, and 10¼ inches deep (inside measurements), and has two shelves. Heat source is a 40-watt light

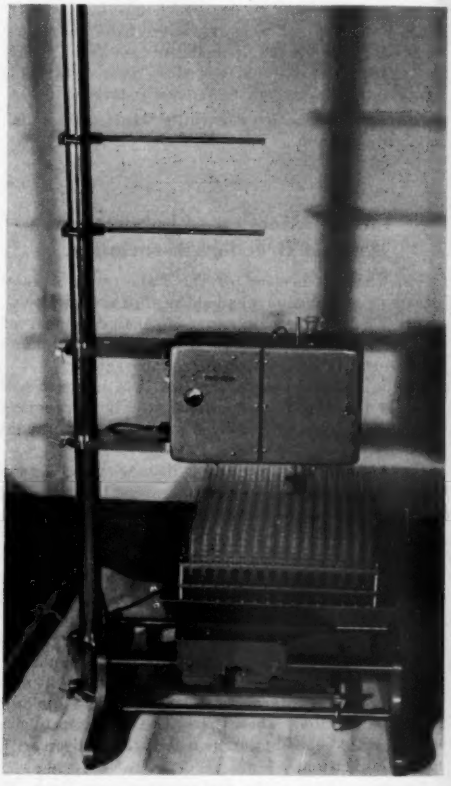


bulb. A heat baffle plate eliminates hot spots. The positive action double brass wafer thermostat has precision of 1 degree Centigrade.

The incubator is constructed of heavy gauge sheet steel and has a removable steel bottom plate to facilitate cleaning. There is a back vent for circulation and a glass-windowed door with a positive magnetic catch.

Fraction Collecting Device

For fraction collecting, the GME V15[2] Fractionator is available from Gilson Medical Electronics. This device eliminates drop counting by using the improved standard volumetric unit which provides a constant volume output per sample.



When a collecting tube is filled with fluid, light from a fluorescent source is focused on an hermetically sealed photocell by the cylindrical lens formed when the sample fills the collecting tube. Interchangeable collecting tubes permit any volume from 1 to 30 cc. to be collected.

Fractions are collected directly, in boustrophedonic order, in removable, rectangular test tube racks, eliminating test tube transfers. Three test tube racks are supplied; each rack holds five rows of 15 test tubes. Racks are available in stainless steel or aluminum and can be used for washing or storing test tubes.

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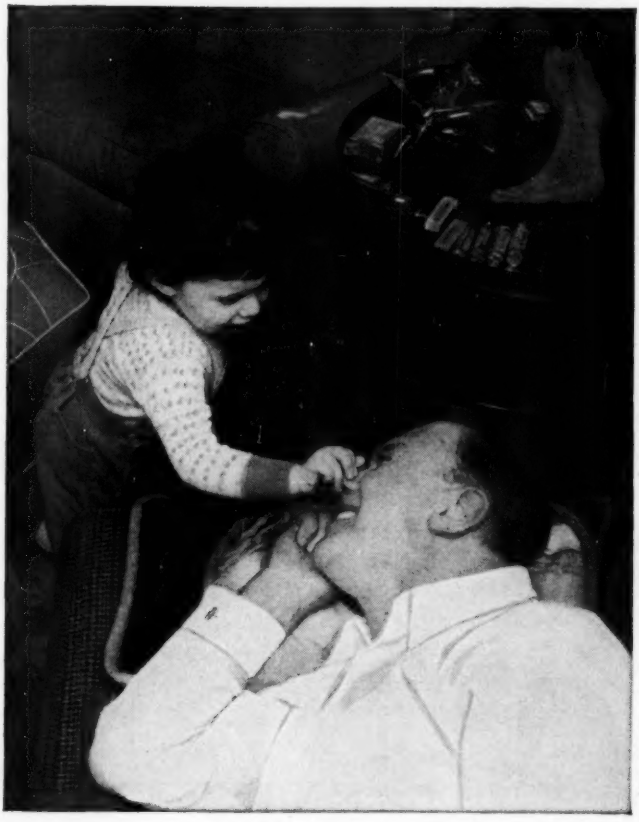

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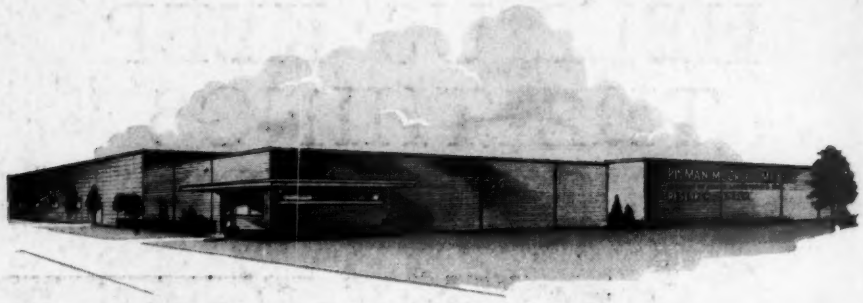

new research facilities for

PITMAN-MOORE COMPANY

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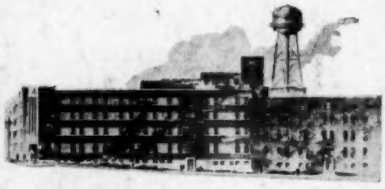
A $1,500,000 building, completed early in 1959, enables Pitman-Moore Company to concentrate in one location all types of research contributing to the development of human and veterinary medical preparations. This new unit adds 41,000 square feet of space for laboratories, offices, and technical equipment in the company's Research Center near Zionsville, Indiana.

The Indianapolis laboratories, where pharmaceuticals are produced.



The Zionsville laboratories, where biologicals are produced.

Within this organization originated the policy: Sales to Graduate Veterinarians ONLY

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